

Supplementary Information

Interkingdom metabolic transformations captured by microbial imaging mass spectrometry

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Biological sciences: microbiology

A) Methods

Strains and materials

Aspergillus fumigatus Af293¹ was a gift from JCVI (Rockville, MD, USA). *Pseudomonas aeruginosa* PA14² came from the D. Hung lab (Harvard Medical School, USA) and originated from F.M. Ausubel's lab (Massachusetts General Hospital, USA). All chemicals used for ISP2 and YES media were purchased from Sigma-Aldrich. Organic solvents are purchased from J. T. Baker. Supelco Discovery DSC-18 20 ml SPE columns were obtained from Sigma-Aldrich. Universal MALDI matrix was purchased from Sigma-Aldrich. 1-hydroxyphenazine (1-HP; **2**) ($\geq 95\%$ GC) and 1-methoxyphenazine (1-MP; **4**) ($\geq 93\%$ GC) were purchased from TCI. Pyocyanin (PYO; **1**) ($\geq 98\%$ by HPLC) and 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS; **11**) ($\geq 96\%$ by HPLC) were purchased from Sigma-Aldrich. Phenazine-1-carboxamide (PCN; **13**) (95% by NMR) and phenazine-1-carboxylic acid (PCA; **3**) (95% by NMR) were purchased from Princeton Biomolecular Research, Inc.

General HPLC conditions

All HPLC purifications were performed on an Agilent infinity 1260 HPLC equipped with a diode array detector, a manual injector, and a Biorad Model 2110 fraction collector. An analytical column (Luna C-18, 250 X 4.6 mm, 5 μ m, Phenomenex) or a semi-preparative column (C-18, 250 X 10 mm, 5 μ m, Supelco) was used for sample analysis and purification at 25°C. For the mobile phase, specified gradients of solvent A (H₂O containing 0.1% TFA) and solvent B (acetonitrile containing 0.1% TFA) were used.

HPLC Purification of commercial phenazines used in this study

HPLC chromatography and the information provided by the commercial suppliers indicated that the commercial phenazines were not pure, therefore we purified them by HPLC. 1-HP (**2**) (1-2 mg / 150 µL MeOH, each run), PCA (**3**) (1 mg/ 400 µL MeOH/AcN = 1/1, each run), 1-MP (**4**) (1-2 mg /150 µL MeOH, each run), and PCN (**13**) (1 mg/400 µL, MeOH/AcN= 1/1, each run) were purified by HPLC (C-18, 250 X 10 mm, 2ml/min) with a gradient of 40 to 100% B in 25 min.

Preparation of bacterial and fungal samples

*Co-culture and mono-culture of *P. aeruginosa* and *A. fumigatus**

A. fumigatus spore stock was prepared by transfer of a fungal slant to YES agar (20 g agar, 150 g sucrose, 20 g yeast, 0.5 g MgSO₄.7H₂O, 5 mg CuSO₄.5H₂O, 10 mg ZnSO₄.7H₂O in 1L DI water). After incubation for 7 days at 28°C, spores were collected in 20% glycerol/water, CFU was adjusted to 7.2e6/ml and the spore stock was stored at -80°C. No tween detergent was used due to anticipated interference with mass spectrometry analysis. *P. aeruginosa* was grown from a single colony in LB overnight to stationary phase (OD= 1.2), diluted to a 20% glycerol/water stock, and stored in small aliquots at -80°C, CFU 1.6e8/ml). For the MALDI IMS experiments this frozen stock was used directly.

P. aeruginosa (CFU 1.6e6) was inoculated at 5 mm distance from an *A. fumigatus* inoculum (CFU 7.2e4) on ISP2 agar (10 mL) in 100 O.D. x 25 mm Petri dishes (Fisherbrand). Similarly, *P. aeruginosa* and *A. fumigatus* were inoculated separately as controls. Samples were incubated for 12h, 24h, 36h and 48h at 30°C. A region of agar was cut covering both

organisms or the singly grown organism (ranging from 2 cm by 2 cm to 3 by 4 cm) and laid on top of a MALDI MSP 96 anchor plate.

Interaction of *A. fumigatus* with phenazines for IMS studies

An *A. fumigatus* inoculum (CFU 7.2 e4) on ISP2 agar was incubated for 24h at 30°C. Paper disks (6 mm diameter) were impregnated with 0.04 µmol of PYO (1) (in EtOH), 1-HP (2) (in EtOH), PCA (3) (in AcN), 1-MP (4) (in EtOH), PCN (13) (in AcN/EtOH= 1/1), PYO (1)+ PCA (3) (in EtOH and AcN respectively), or control solvents. The paper disks were allowed to air dry and placed adjacent to the outer edge of the pre-grown fungal colony. In addition, paper disks impregnated with the same amount of phenazines were placed on blank ISP2 agar. After incubation for an additional 24 h at 30°C a region of the agar containing the fungus and the paper disk was cut and transferred to a MALDI target plate. On the same MALDI plate an agar section containing the fungus grown adjacent to a blank paper disk was placed, in addition to a section of the agar containing the paper disk impregnated with control phenazine(s). All paper disks were removed prior to subsequent steps in sample preparation.

MALDI-IMS

A photograph was taken of the agar sections transferred to a MALDI MSP 96 anchor plates and the aerial hyphae of *A. fumigatus* were subsequently gently removed with a cotton swab dampened in acetonitrile. Another photograph was taken and a thin layer of universal matrix was applied to the sample using a 53 µm molecular sieve. Samples were dried at 37°C for a minimum of 5 h.

The timecourse samples of *P. aeruginosa* – *A. fumigatus* interactions and the controls were subjected to Autoflex Bruker Daltonics MALDI-TOF MS for imaging MS acquisition and

the samples were run in positive reflectron mode, with 400 µm- 600 µm laser intervals in XY and a mass range of 100-3000 Da. The fungal-phenazine samples were subjected to Microflex Bruker Daltonics MALDI-TOF MS for imaging MS acquisition and these samples were run in positive reflectron mode with 400-600 µm laser intervals in XY and a mass range of 100-2000 Da. The data on both instruments were analyzed using FlexImaging 2.0 software. Detailed instrument parameters for collecting image data were described in reference 3. Intensity of metabolite ions in MALDI-IMS depends on various factors including concentration, and ionization efficiency.

For the MALDI FT-ICR IMS of a 48h co-culture of *P. aeruginosa* and *A. fumigatus* and their controls mass spectra were acquired using a 9.4 T Apex Qe (Bruker Daltonics, Billerica MA) equipped with an Apollo II dual ion source and a 355 nm solid state Smartbeam laser focused to a diameter of 100 µm. Data were acquired and analyzed using the FlexImaging application with a pixel spacing of 450 µm for plate A and 150 µm for plate B. The mass spectrum at each pixel was accumulated from 3 scans of 200 laser shots each. The scanned mass range was 100-600 Da.

General extraction protocols for MS analysis

To identify the ions of interest observed in the MALDI-IMS, co-cultures of *P. aeruginosa* and *A. fumigatus* (as described above) and singly grown organisms were subjected to a variety of extraction protocols and analyzed by MS and MS/MS network analysis.

- 1) Ten ISP2 agar plates were inoculated each with two *P. aeruginosa* - *A. fumigatus* interactions or singly grown organisms as described above. The plates were incubated for 48h at 30°C and rectangles were cut containing the co-cultures, sliced in small pieces, and

placed in 20 mL glass vials. The agar was extracted with EtOAc (2x 10 mL), and subsequently with MeOH (2X 10 mL). Both organic layers were kept separately and were concentrated in vacuo, resuspended in MeOH and centrifuged. The EtOAc extract and the subsequent MeOH extract were each fractionated by HPLC (C-18, 250 x 10 mm, 2 ml/min) with a gradient of 10 to 100% B in 35 minutes and fractions were analyzed by MS.

- 2) Four ISP2 agar plates inoculated with two *P. aeruginosa* - *A. fumigatus* interactions or singly grown organisms as described above, were incubated at 30°C for 48 h. Rectangles containing both organisms were excised, cut in small pieces, and extracted twice with nBuOH (8 ml each). The organic solvent was concentrated in vacuo, the residue resuspended in MeOH and centrifuged. The supernatant was subjected to MS and data dependent MS² analysis and the data was converted to MS/MS networks as described below.
- 3) Small agar plugs (diameter 3 mm) were removed from the interface of a *P. aeruginosa* - *A. fumigatus* interaction (48h, 30°C ISP2). Either 200µL of H₂O/0.1% formic acid, or 35% H₂O/65% AcN/0.1% formic acid was added and the sample was allowed to stand for 1.5 h at rt. The aqueous extracts were centrifuged and analyzed directly by MS.
- 4) To study the relative amounts of 1-HP (**2**) in a *P. aeruginosa* – *A. fumigatus* interaction by HPLC, 4 co-cultures were grown on separate plates. Rectangles were cut in the agar either on the fungal side, the interaction side, or the *P. aeruginosa* side and extracted with EtOAc (3X 10 mL). The extraction solvent was removed under vacuum and the residue taken up in MeOH (100- 500 µL), centrifuged and analyzed by HPLC (C-18, 250 X 4.5 mm, 1 ml/min) with a gradient of 40 to 55% B in 15 minutes followed by 55% to 100% B in 10 min.

5) For comparison of the phenazine biotransformations by *A. fumigatus*, 4 fungal colonies, each grown adjacent to a paper disk impregnated with a phenazine or a blank control as described above, were extracted with nBuOH (3 x 5mL). The solvent was removed under vacuum and the residue resuspended in MeOH, centrifuged, and analyzed by MS and HPLC (C-18, 250 x 4.6 mm, 1 ml/min) with a gradient of 10 to 100% B in 35 min.

Purification of 1-phenazine sulfate (5)

A set of 25 ISP2 agar plates was inoculated with four *P. aeruginosa* - *A. fumigatus* interactions per plate as described above. The plates were incubated for 48h at 30°C and rectangles were cut corresponding to the area observed in the MALDI-IMS to contain m/z 277 Da (Figure 2 of the main article), sliced in small pieces, and treated with EtOAc (50 mL). After sonication for 10 min the mixture was allowed to stand at rt for 2 h. The organic solvent was removed and the process was repeated twice. MeOH (50 ml) was added, the mixture was sonicated for 10 min and allowed to stand at rt for 2h. After removal of the MeOH layer this process was repeated twice. The combined MeOH layers were concentrated under vacuum to a small volume, centrifuged and the supernatant was applied to a prewashed and equilibrated (water/0.1%TFA) Supelco Discovery DSC-18 20 ml SPE column. After washing with water/0.1%TFA, and elution with 30% AcN/water/0.1%TFA, the eluent was concentrated to a small volume and subjected to HPLC analysis (C-18, 250 x 10 mm, 2 ml/min) with a gradient of 10 to 100% B in 35 min to yield 300 µg of phenazine-1-sulfate (**5**) which was used immediately for further studies.

Isolation of 1-MP (4**) from an inoculation of 1-HP (**2**) with *A. fumigatus***

To 10 tubes with 5 ml of ISP2 liquid culture *A. fumigatus* spore stock (2.2e6 CFU each tube) was added. The tubes were shaken for 24 h at 30°C and combined into a 250 ml liquid ISP2 broth and shaken for another 24 h at 30°C. 5 mg of 1-HP (**2**) in 250 µL DMSO was added and the fungal culture was shaken for 4d at 30°C. The entire batch of liquid media was applied to a Supelco Discovery DSC-18 20 ml SPE column (equilibrated with water/0.1% TFA). The column was subsequently washed with water/0.1% TFA, followed by 30% AcN/water/0.1% TFA and the desired product was eluted with 100% AcN/0.1% TFA. The eluent was concentrated in vacuum to yield 14.5 mg of material, which was dissolved in MeOH (1 mL) and purified by HPLC (C-18, 250 x 10 mm, 2 ml/min) with a gradient of 20 to 100% B in 35 min to yield 700 µg of 1-MP (**4**).

General MS procedures for extract analysis

For the ion trap and FT-ICR MS data acquisition, each extract or compound was dissolved in spray solvent 50:50 MeOH/H₂O containing 1% formic acid, and underwent nanoelectrospray ionization on a biversa nanomate (Advion Biosystems, Ithaca, NY) using a back pressure of 0.3-0.5 p.s.i. and the spray voltage of 1.3 -1.45 kV. MS and MS/MS spectra were acquired on a 6.42 T Finnigan LTQ-FT-ICR MS or a Finnigan LTQ-MS (Thermo-Electron Corporation, San Jose, CA) running Tune Plus software version 1.0 and Xcalibur software version 1.4 SR1. The instrument was first autotuned on the *m/z* value 816 of a cytochrome C. Ions of interest were isolated by the linear ion trap and fragmented by collision induced dissociation (CID). Isolation window for ion trap was 1-2 *m/z* and for FT-ICR 2-3 *m/z*. Collision energy was 35kV for both ion trap and FT-ICR.

To generate MS/MS networks, tandem MS data were gathered by infusion of the extracts into the mass spectrometer using the Nanomate nanospray source coupled to the ion trap. A preprogrammed data acquisition method was used that was generated with the instrument's controlling software: Xcalibur 1.0 (Thermo Electron Corp.). This data acquisition method involved repeatedly acquiring a single low-resolution MS¹ scan followed by data-dependent CID (MS²) of the top five most abundant ions from the MS¹ scan. Each ion was trapped and fragmented three times before being added to an exclusion list. The exclusion list size was set to 500 ions, with an exclusion duration of 10 min during the acquisition method. The *m/z* isolation width was set to 2, activation Q at 0.25, the activation time set to 100ms, and the activation energy set to 35%. In addition, a FT-ICR full scan of the extract was acquired by infusion into the mass spectrometer using the Nanomate nanospray source.

NMR measurement

Phenazines were dissolved in 50 µL of CD₃OD for NMR acquisition. NMR spectra were recorded on Bruker Avance III 600 MHz NMR with 1.7 mm Micro-CryoProbe at 298 K, with standard pulse sequences provided by Bruker. Data was analyzed using the Topspin 2.1 software.

Bioassay

Inhibition of *A. fumigatus* growth was assessed with an agar disk diffusion assay: *A. fumigatus* spore stock (CFU 1.4 e5) was spread over 100 O.D. x 25 mm Petri dishes (Fisherbrand) using glass beads. Paper disks (6 mm) were treated with escalating doses of PYO (**1**) (in EtOH), 1-HP (**2**) (in EtOH), 1-MP (**4**) (in EtOH), PCA (**3**) (in AcN/EtOH), PCN (**13**)

(in AcN/EtOH), phenazine-1-sulfate (**5**) (in EtOH), or control solvents. The disks were allowed to air dry and placed on the fungal lawn. After incubation at 30°C for 48 h diameters of clearance zones were measured in whole mm.

Construction of MS/MS spectral networks

Tandem mass spectra were clustered according to the protocol described by Watrous et al⁴ with a slight modification; all algorithms assumed precursor mass tolerance of 0.9 Da and fragment mass tolerance of 0.3 Da.

Identification of ions within MS/MS networks

Ions of interest observed in low resolution MALDI-TOF IMS (microflex and autoflex) were correlated to high resolution MALDI FT-ICR IMS (*P. aeruginosa*- *A. fumigatus* interaction at 48h), and MS/MSⁿ data collected with an ion trap and FT-ICR on corresponding extracts. Data dependent MS² of the extracts was converted into a MS/MS network and ions of interest and their relationship to other clustered ions was investigated. FT-ICR MS data was used to obtain a positive ID for at least one ion in a particular cluster by searching against databases including the Antimarin database, dictionary of natural products and SciFinder. Identification was verified by analysis of the MSⁿ data, comparison with literature data and commercially available materials. Identities of other ions in the cluster were deduced by comparison of MS² fragmentation patterns and literature data if available. This process simplified the identification process of analogs of molecules that are not listed in currently available databases.

B) Figures

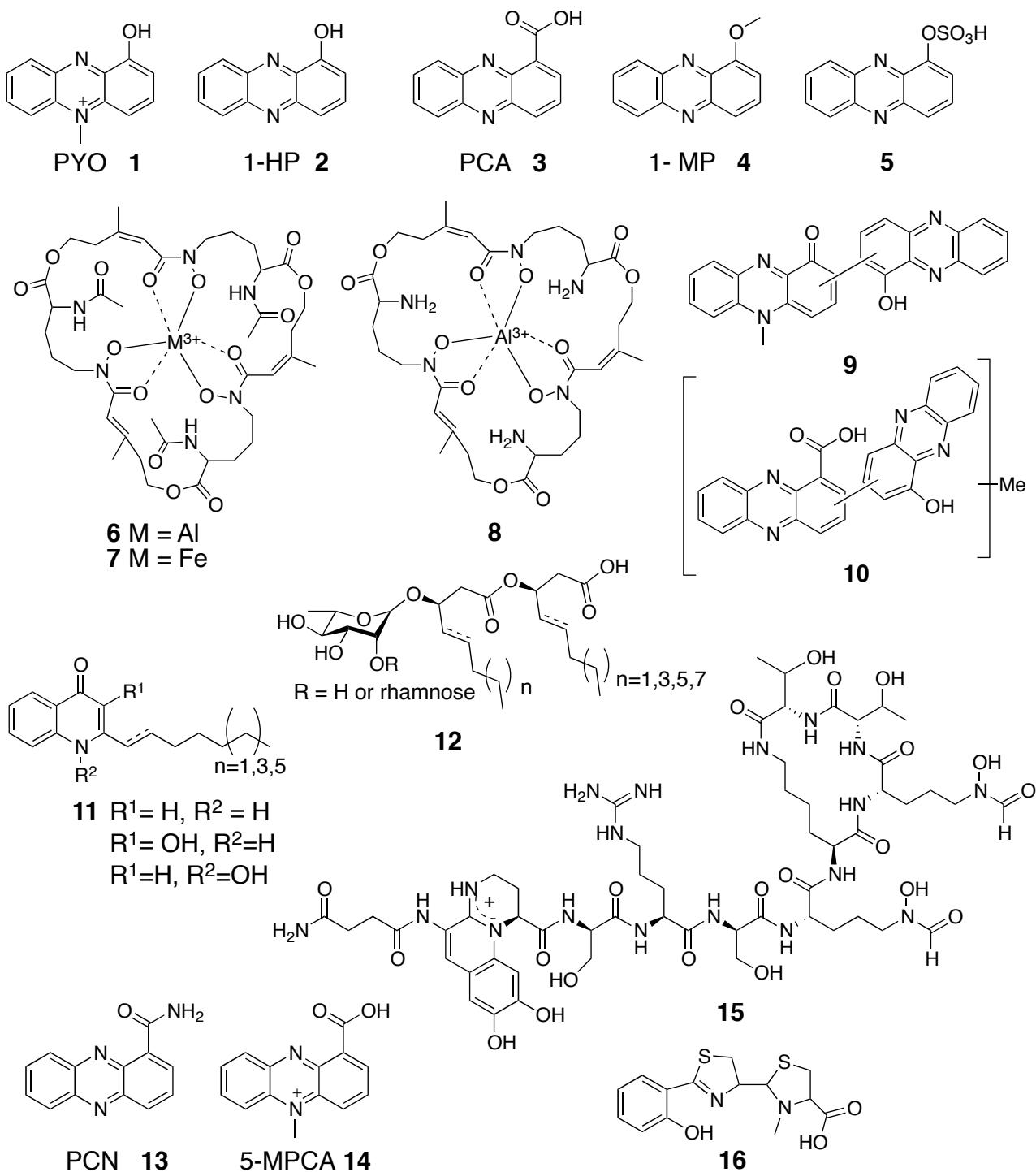


Figure S1. *P. aeruginosa* and *A. fumigatus* metabolites observed with MALDI-IMS described in this paper. Phenazines (**1-5**, **13-14**), quinolones (**11**, double bond location not determined), monorhamnolipids (**12** R=H, double bond location not determined) and dirhamnolipids (**12** R=Rha, double bond location not determined), *P. aeruginosa* siderophores pyoverdin E (**15**) and pyochelin (**16**) and the metal bound fungal siderophores triacetyl fusarinine C and fusarinine C (**6**, **7**, **8**). Structures of phenazine-dimers (**9,10**) are putative and based on MSⁿ fragmentation.

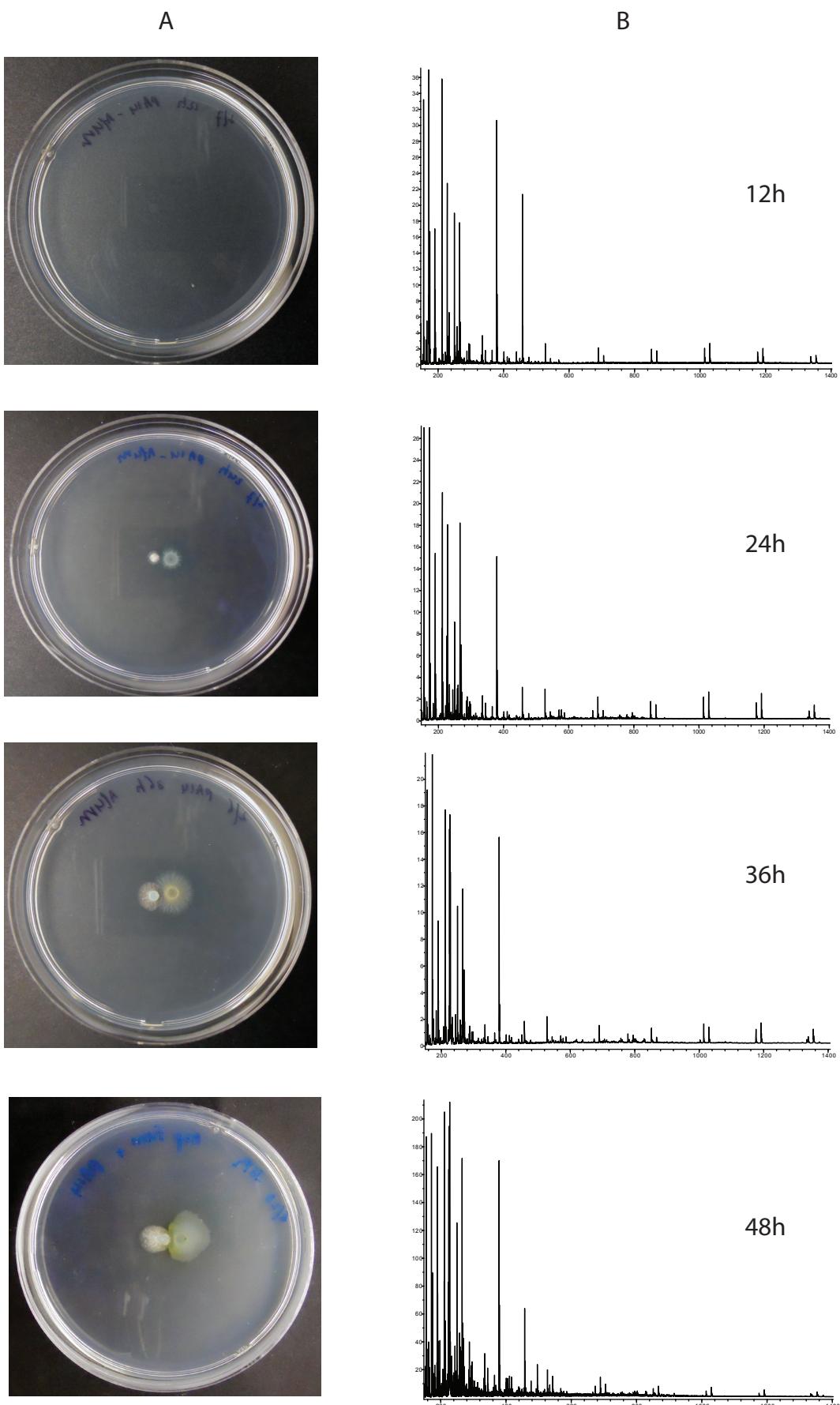


Figure S2. (A) Interaction of *A. fumigatus* (left) and *P. aeruginosa* (right) at 12h, 24h, 36h and 48h. (B) average MALDI-IMS spectra (mass range 100 – 3000 Da) acquired on a Bruker Autoflex.

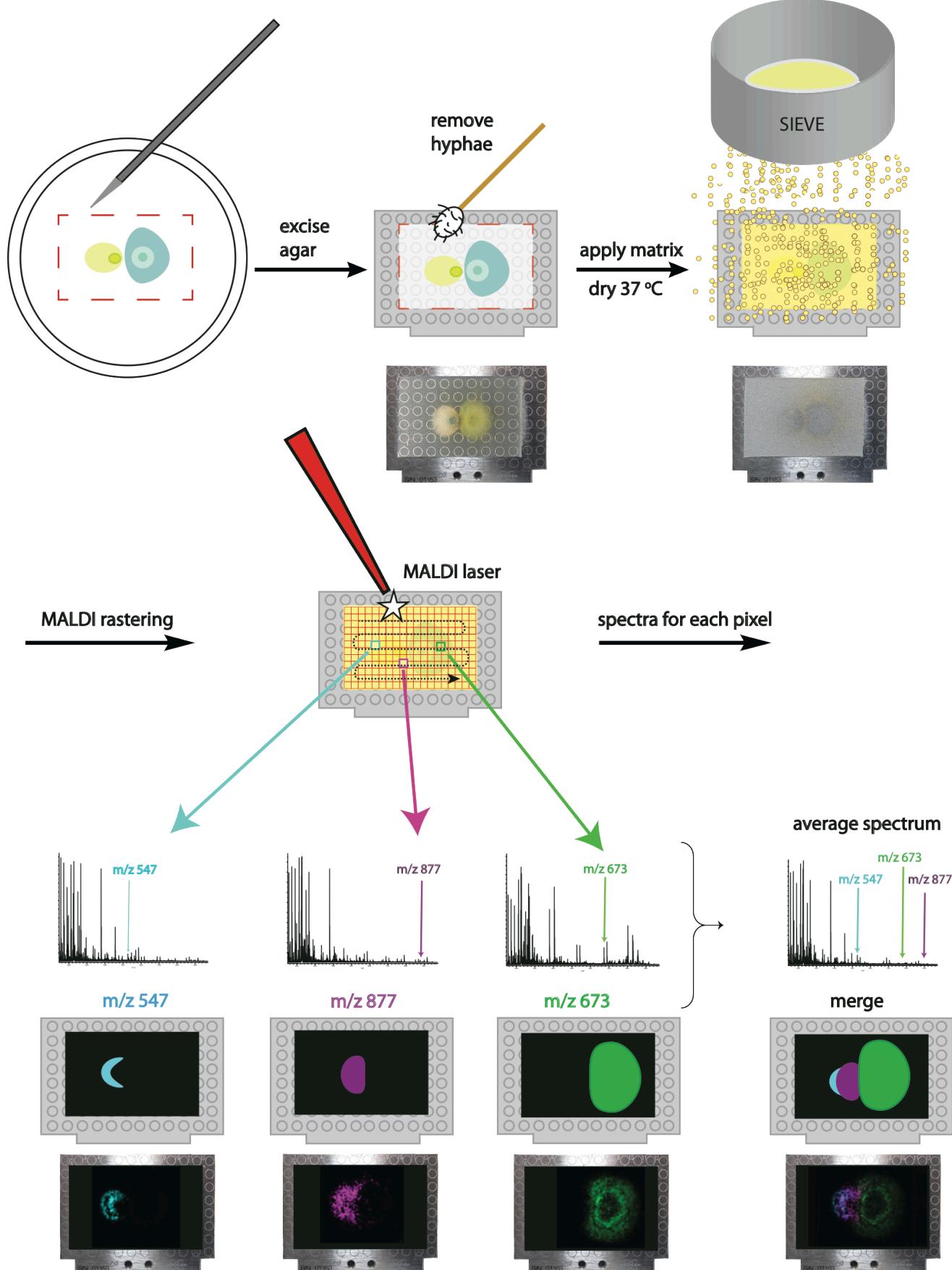


Figure S3. General overview of sample preparation and MALDI imaging.

A. fumigatus and *P. aeruginosa* are grown on ISP2 agar in a petridish, excised, layered on a stainless steel MALDI plate and a photograph is taken. Aerial hyphae are removed with a cotton swab dipped in acetonitrile, universal matrix is applied through a $53\text{ }\mu\text{m}$ sieve and the sample is dried at 37°C . MALDI mass spectra are obtained for each pixel in the area of interest and averaged. Ions with specific distributions are visualized using a false color.

A

B

IMS data acquired on a Bruker FT-ICR

quinolone (11)
C9:db-PQS/C9:db-NQNO
 m/z 286.1799

quinolone (11)
PQS/HQNO
 m/z 260.1657

rhamnolipid (12)
Rha-Rha-C10-C10
 Na^+ salt m/z 673.3794

pyochelin (16)
 m/z 325.0669

pyoverdin E (15)
 m/z 1333.6

fungal product
 m/z 547.2316

PCN (13)
 m/z 224.0810

PYO (1)
1-MP (4)
 m/z 211.0855

PCA (3)
 m/z 225.0652

1-HP (2)
 m/z 197.0716

5-MPCA (14)
 m/z 239.0813

phenazine-1-sulfate (5)
 m/z 277.0278

phenazine dimer (9)
 m/z 405.1340

phenazine dimer (10)
 m/z 433.1290

triacytlyfusarinine C [Al³⁺] (6)
 m/z 877.3712

triacytlyfusarinine C[Fe³⁺] (7)
 K^+ salt m/z 944.3

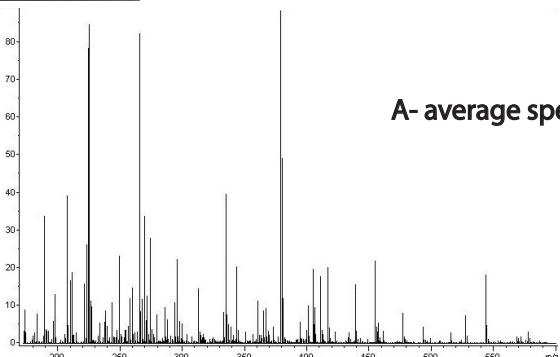
fusarinine C[Al³⁺] (8)
 K^+ salt m/z 789.3

NA = not analyzed

excised agar

hyphae removed

matrix applied



excised agar

hyphae removed

matrix applied

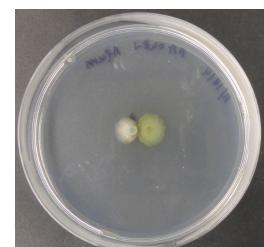
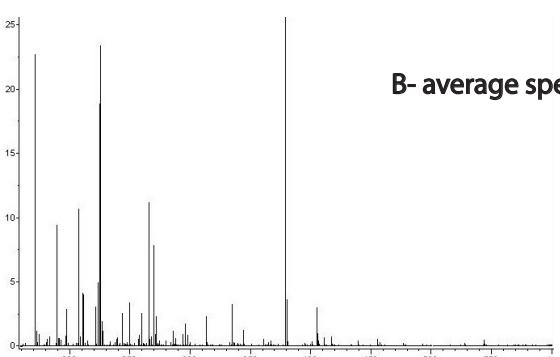


Figure S4. Duplicate MALDI FT-ICR imaging data of interaction between *A. fumigatus* and *P. aeruginosa* (ISP2, 48h, 30°C). The detected mass range was 100- 600 Da. Image area of column A includes both organisms with a pixel spacing of 450 μ m, while column B was a strip through both organisms with a pixel spacing of 150 μ m. Different stages of sample preparation and average spectra are also shown. 14

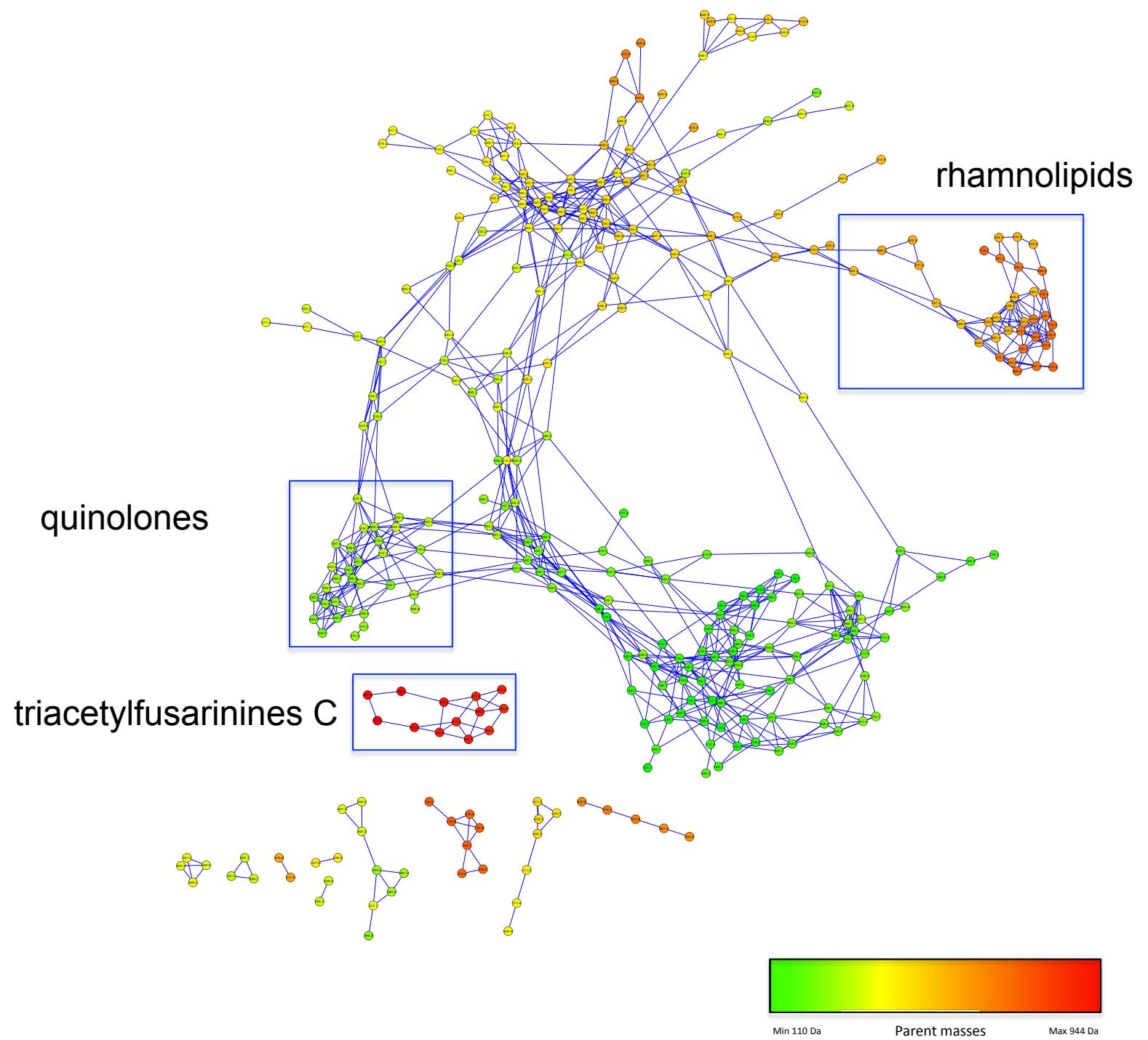
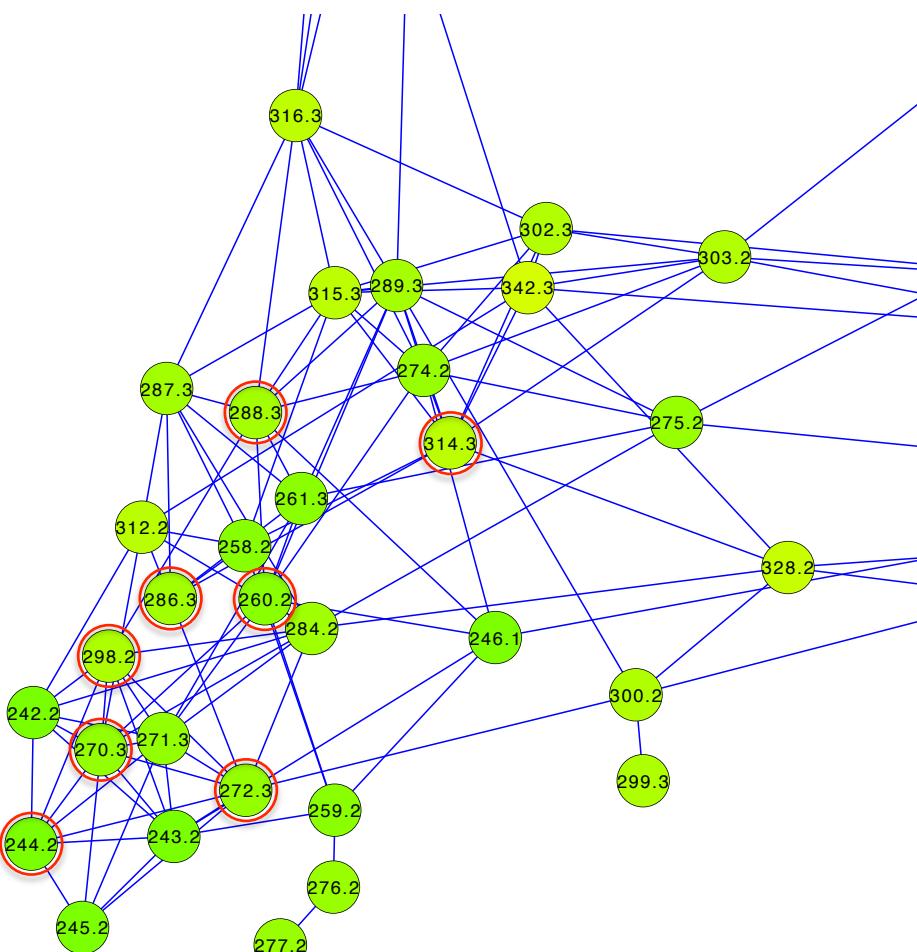


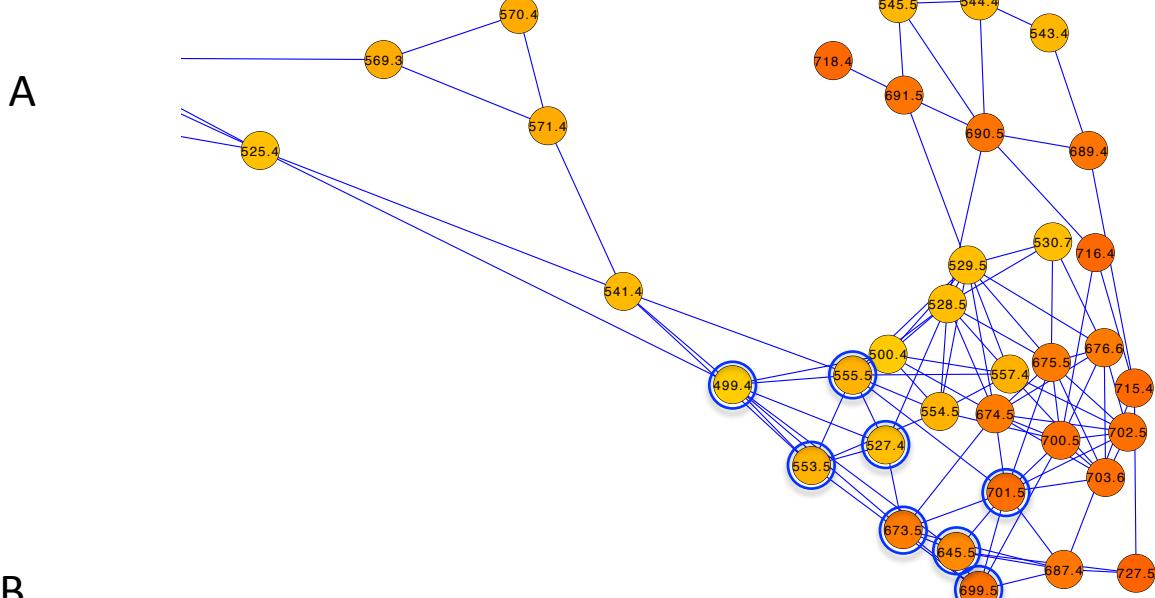
Figure S5.1 MS/MS network of nBuOH extract of *A. fumigatus* and *P. aeruginosa* interaction (ISP2, 48h, 30°C) highlighting different metabolite classes



B

m/z	Formula	Form	Name	H⁺ Calc.	H⁺ Obs	error ppm
244	C ₁₆ H ₂₁ NO	H ⁺	HHQ	244.1696	244.1683	-5.3
260	C ₁₆ H ₂₁ NO ₂	H ⁺	PQS, HQNO	260.1646	260.1641	-1.9
270	C ₁₈ H ₂₃ NO	H ⁺	C9:db NHQ	270.1853	270.1843	-3.7
272	C ₁₈ H ₂₅ NO	H ⁺	NHQ	272.2009	272.2009	0.0
286	C ₁₈ H ₂₃ NO ₂	H ⁺	C9:db-PQS, C9:db-NQNO	286.1802	286.179	-4.2
288	C ₁₈ H ₂₅ NO ₂	H ⁺	C9-PQS, NQNO	288.1958	288.1947	-3.8
298	C ₂₀ H ₂₇ NO	H ⁺	C11:db UHQ	298.2166	298.2154	-4.0
314	C ₂₀ H ₂₇ NO ₂	H ⁺	C11-PQS, UQNO	314.2115	314.2103	-3.8

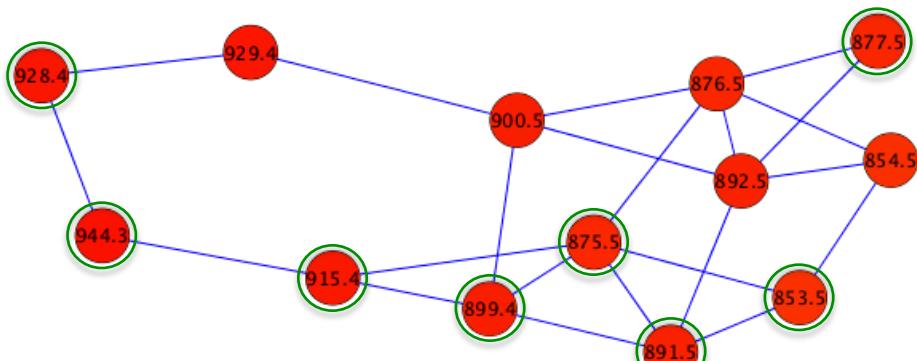
Figure 5.2 (A) Quinolone cluster in nBuOH extract of *A. fumigatus* and *P. aeruginosa* interaction displaying parent masses and their ^{13}C isotopes. (B) Examples of quinolone ions observed in both MALDI-IMS and ion trap MS/MS network (circled in red). Accurate masses are based on FT-ICR full scan of the extract. For quinolone nomenclature see reference 5. db = double bond



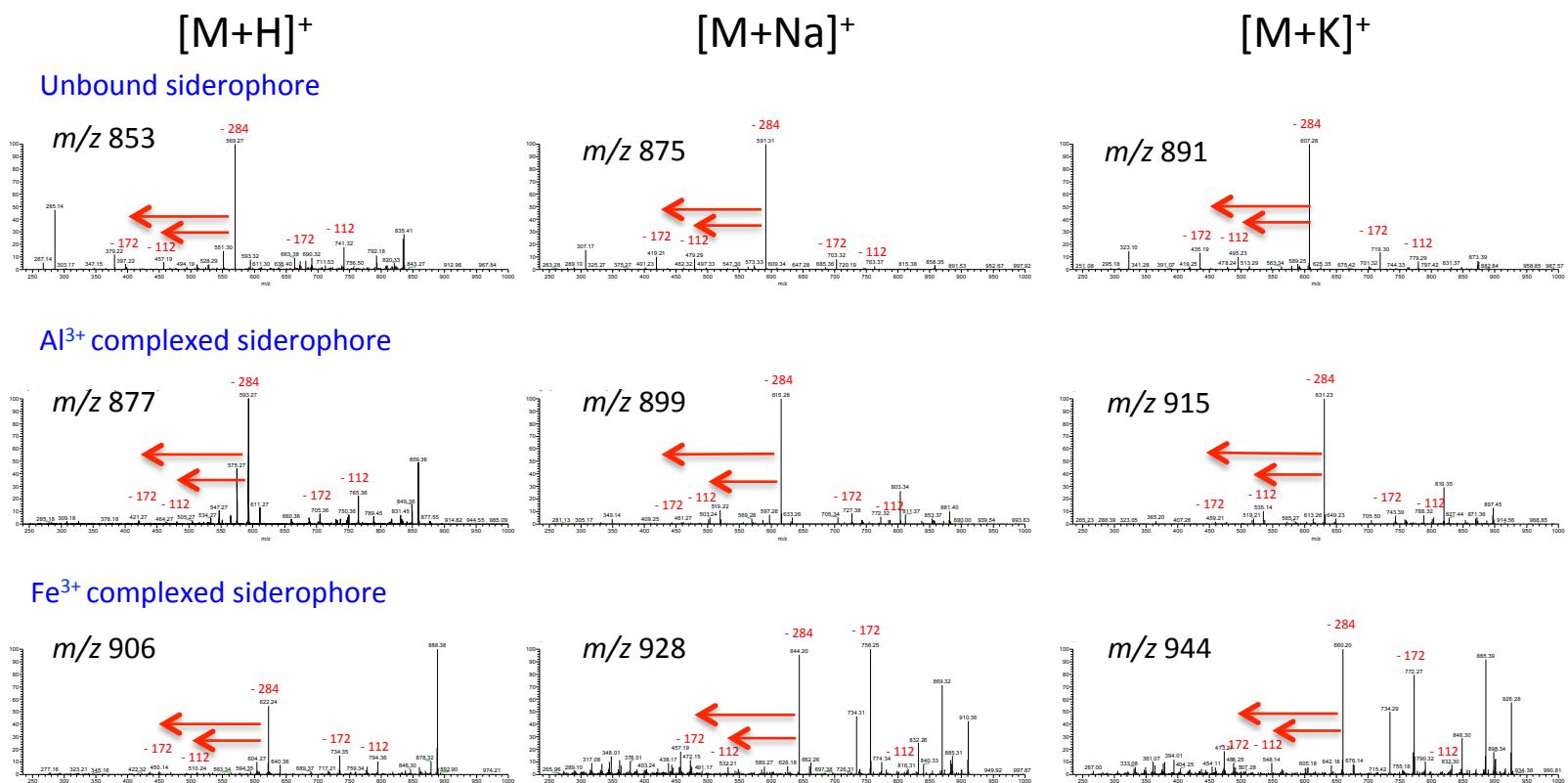
<i>m/z</i>	Formula	Form	Name	Calc mass	Obs exact mass	ppm error
499	$C_{24}H_{44}O_9Na$	Na^+	Rha-C8-C10	499.2878	499.2894	3.2
			Rha-C10-C8			
527	$C_{26}H_{48}O_9Na$	Na^+	Rha-C10-C10	527.3192	527.3204	2.3
			Rha-C10-C10:db			
553	$C_{28}H_{50}O_9Na$	Na^+	Rha-C10-C12:db	553.3347	553.3372	4.5
			Rha-C12:db-C10			
555	$C_{28}H_{52}O_9Na$	Na^+	Rha-C10-C12	555.3504	555.3526	4
			Rha-C12-C10			
645	$C_{30}H_{54}O_{13}Na$	Na^+	Rha-Rha-C10-C8	645.3457	645.3486	4.5
			Rha-Rha-C8-C10			
673	$C_{32}H_{58}O_{13}Na$	Na^+	Rha-Rha-C10-C10	673.377	673.3742	-4.1
699	$C_{34}H_{60}O_{13}Na$	Na^+	Rha-Rha-C12:db-C10	699.3926	699.3962	5.1
			Rha-Rha-C10-C12:db			
701	$C_{34}H_{62}O_{13}Na$	Na^+	Rha-Rha-C12-C10	701.4083	701.4125	5.9
			Rha-Rha-C10-C12			

Figure S5.3 (A) Rhamnolipid cluster in nBuOH extract of *A. fumigatus* and *P. aeruginosa* interaction displaying parent masses and their ^{13}C isotopes. (B) Examples of rhamnolipid ions observed in both MALDI-IMS and ion trap MS/MS network (circled in blue). Only the $[M+Na]^+$ species are listed. Accurate masses are based on FT-ICR full scan of the extract. For rhamnolipid nomenclature see reference 6.

A



B



C

FT-ICR (full scan crude extract)	$[M+H]^+$	formula	Error (ppm)
unbound	853.4122	$\text{C}_{39}\text{H}_{61}\text{N}_6\text{O}_{15}$	-7.8
Al^{3+} complex	877.3698	$\text{C}_{39}\text{H}_{58}\text{N}_6\text{O}_{15}\text{Al}$	-8.2
Fe^{3+} complex	906.3231	$\text{C}_{39}\text{H}_{58}\text{N}_6\text{O}_{15}\text{Fe}$	-8.1

Figure S5.4 (A) Triacetylfusarine C cluster in nBuOH extract of *A. fumigatus* and *P. aeruginosa* interaction displaying parent masses (circled in green) and ¹³C isotopes. (B) MS/MS Spectral comparisons. (C) Accurate masses are based on FT-ICR full scan of the extract.

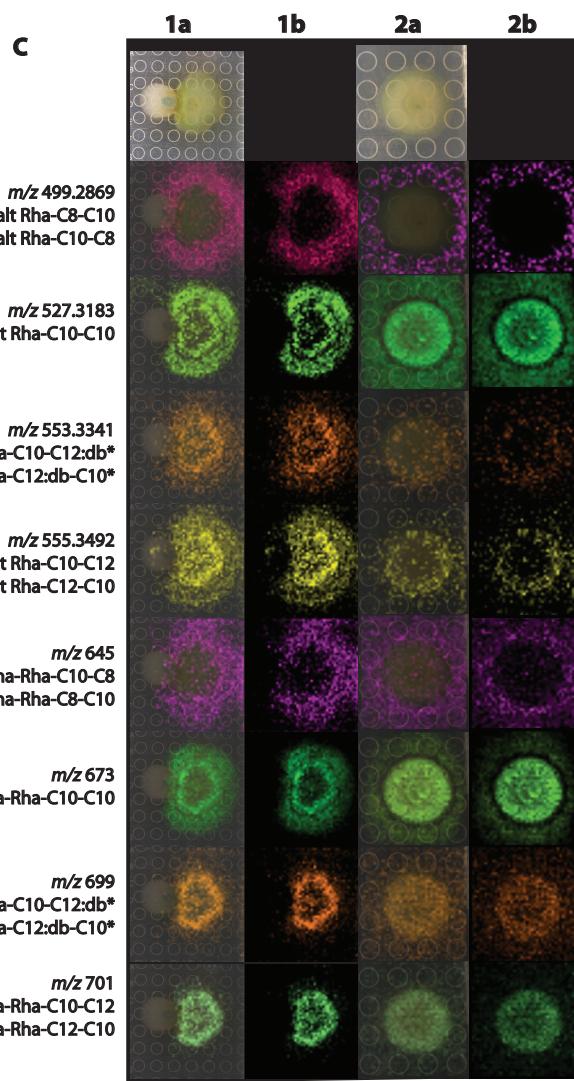
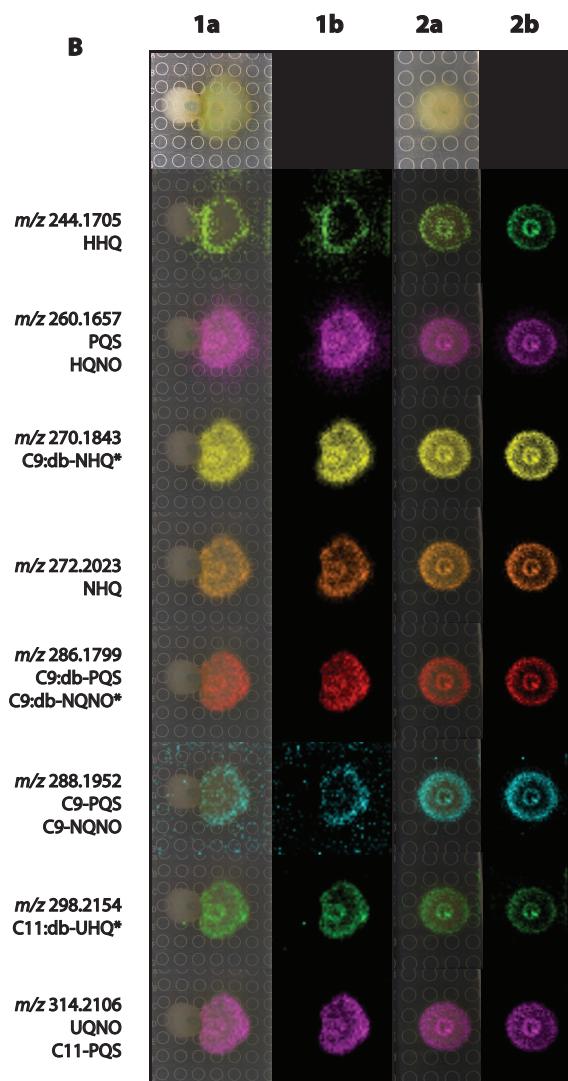
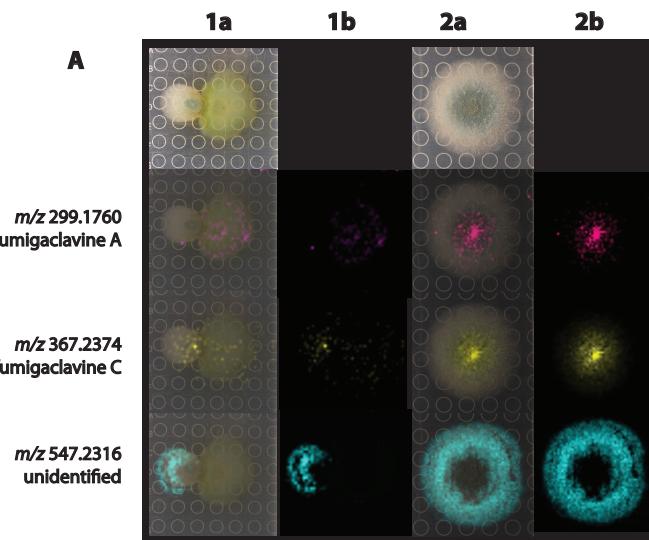


Figure S6. (A) Comparison of fungal metabolites produced by *A. fumigatus* grown in proximity to *P. aeruginosa* vs a control *A. fumigatus* colony (ISP2, 30°C, 48h). (B) quinolones **11**, [M+H]⁺ observed in *P. aeruginosa* grown in proximity to *A. fumigatus* (column 1) vs in control *P. aeruginosa* (column 2) at 48h (30°C). (C) Rhamnolipids (**12**, [M+Na]⁺ observed in *P. aeruginosa* grown in proximity to *A. fumigatus* (column 1) vs in control *P. aeruginosa* (column 2) at 48h (30°C). All columns display ion distributions as false colors. Columns (a) are overlays with the optical image (top panel). Columns (b) are false color only. For the *m/z* 100-600 Da range accurate masses shown are determined by replicate MALDI FT-ICR (Fig. S3) *db = contains one double bond.

For quinolone and rhamnolipid nomenclature see references 5,6.

Diameter clearance zone, nearest whole mm					
μmol	0.02	0.04	0.08	0.16	0.20
PYO (1)	0	0	0	0	0
PCA (3)	0	0	0	0	0
PCN (13)	0	0	discolor	discolor	discolor
1-HP (2)	0	0	discolor	10	10
1-MP (4)	0	0	15	21	21
Phenazine-1-sulfate (5)	0	0	0	0	0

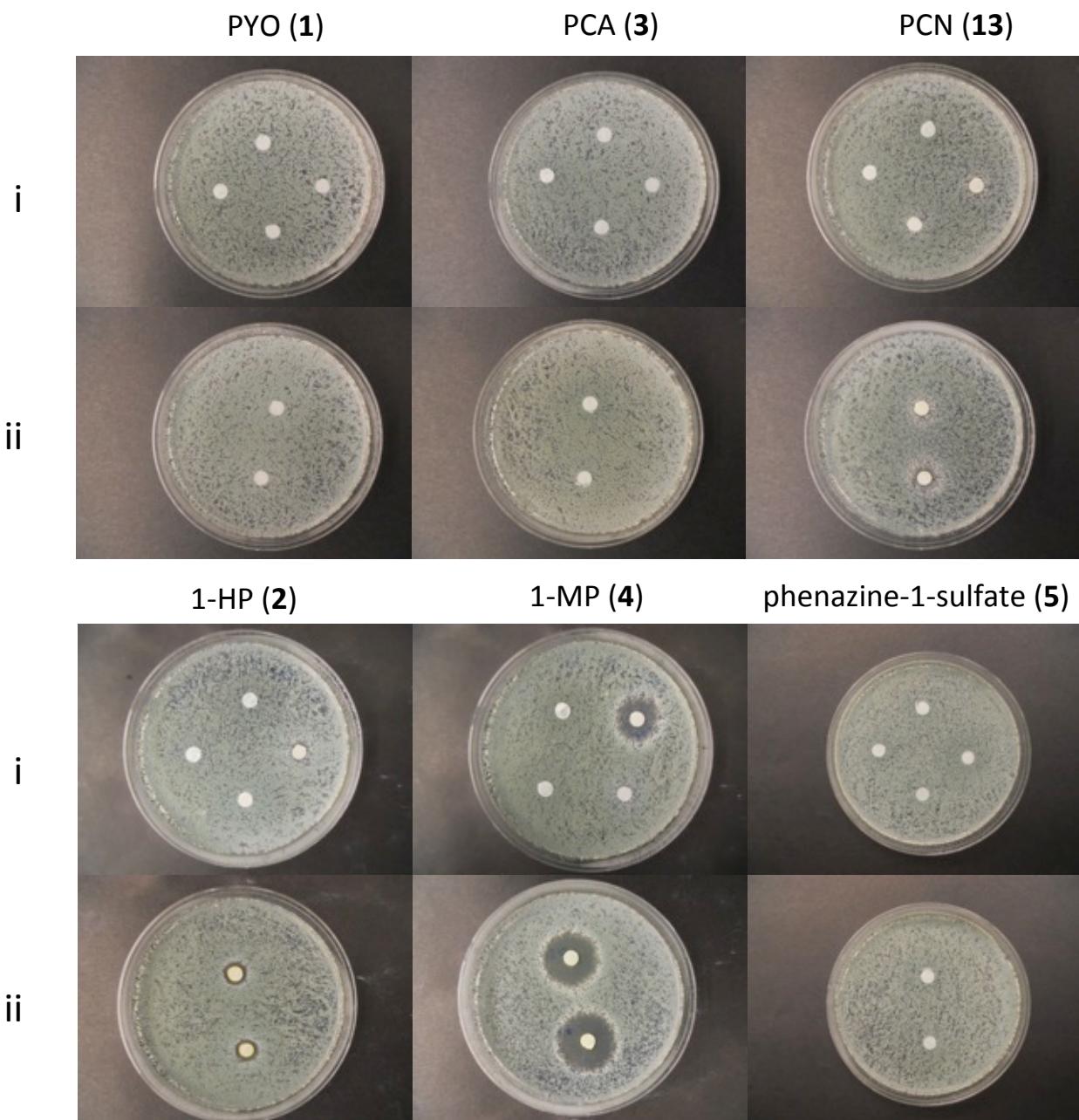
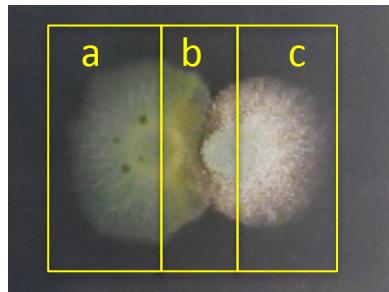


Figure S7. Disk diffusion assay of phenazines on a lawn of *A. fumigatus* (ISP2, 48h, 30°C) (i) counterclockwise starting at the top: blank, 0.02 μmol, 0.04 μmol, 0.08 μmol phenazine (ii) 0.16 μmol (top), 0.20 μmol (bottom) phenazine 20

A



B

5.9 min 7.8 min 8.4 min 9.8 min
PCN 1-MP 1-HP PCA

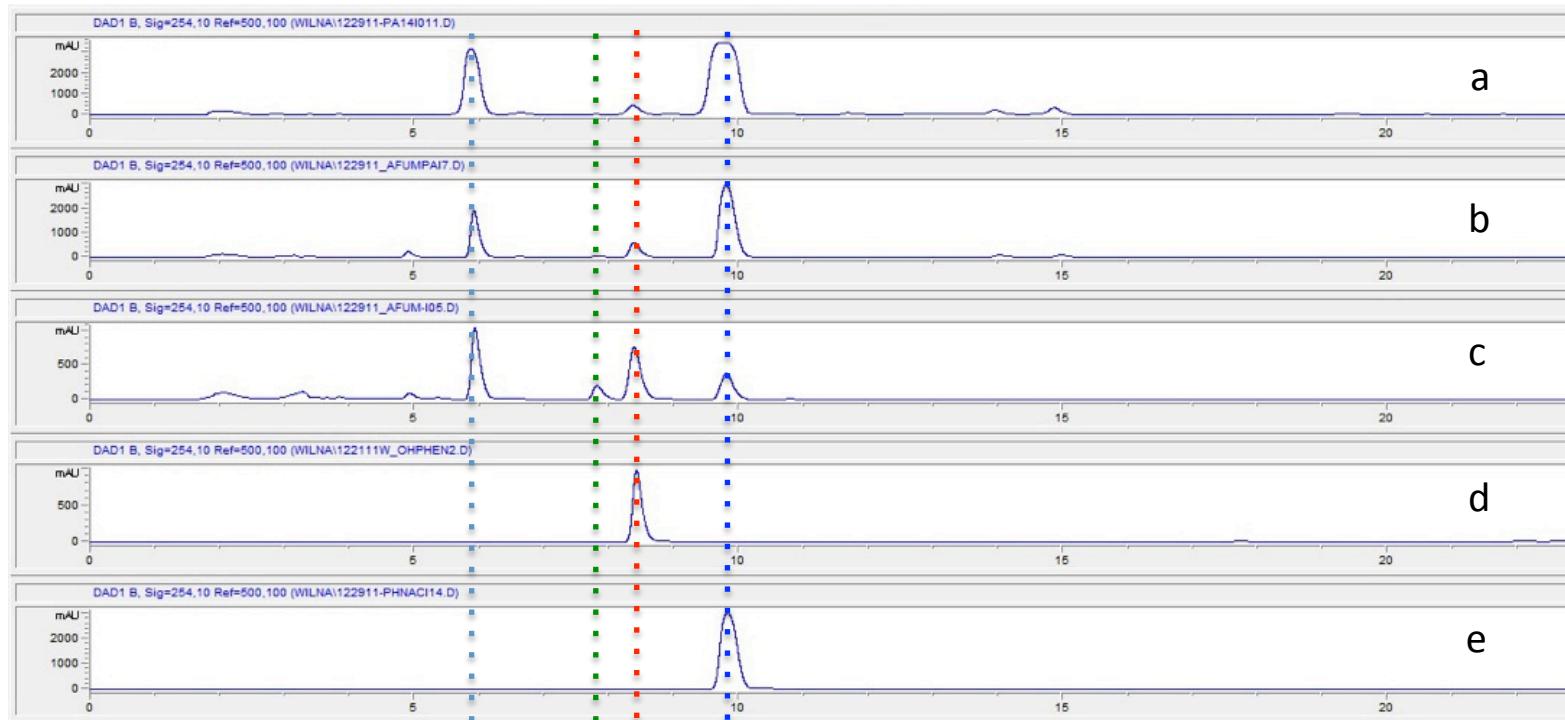
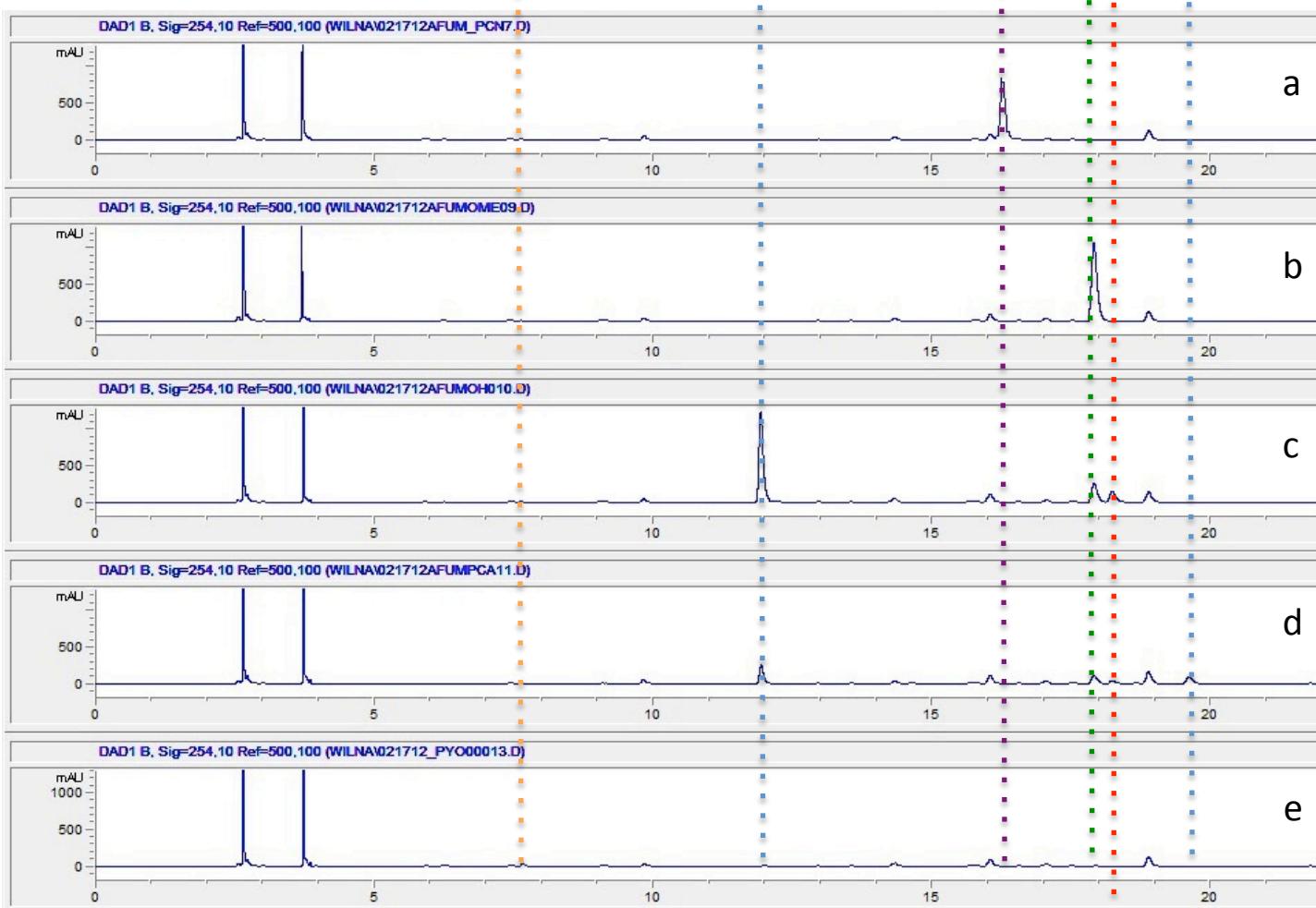


Figure S8.

(A) Interaction of *P. aeruginosa* (left) and *A. fumigatus* (right) on ISP2 (30° , 48h). Areas (a), (b), and (c) were excised, extracted with EtOAc, and analyzed by HPLC.
 (B) HPLC chromatograms (254 nm) of regions (a), (b), and (c) in the interaction compared to phenazine standards. (a) *P. aeruginosa* region outside, (b) Interface *P. aeruginosa* and *A. fumigatus* (c) *A. fumigatus* region outside, (d) standard 1-HP, (e) standard PCA.

HPLC gradient: 40 to 50% ACN in H_2O with 0.1% TFA (15 min), 50 to 100% ACN in H_2O with 0.1% TFA (10 min), hold (3 min), back to starting conditions (2 min).

A

PYO Phenazine-1-sulfate 1-HP
PCN 1-MP PCA

B

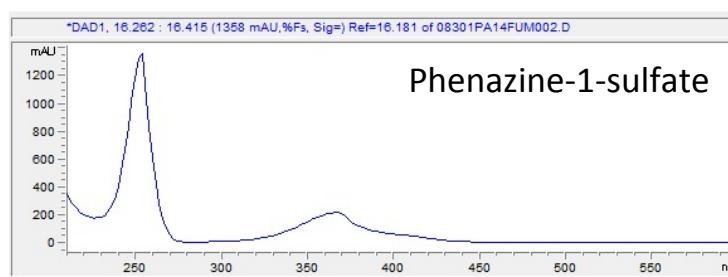
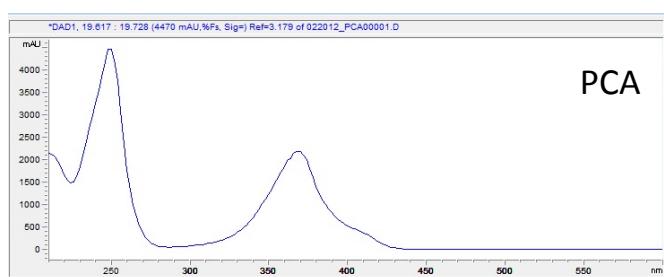
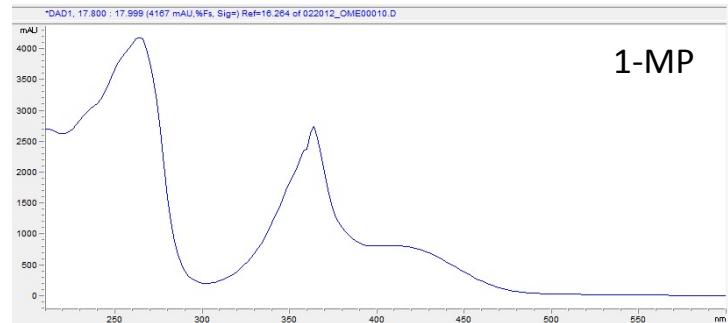
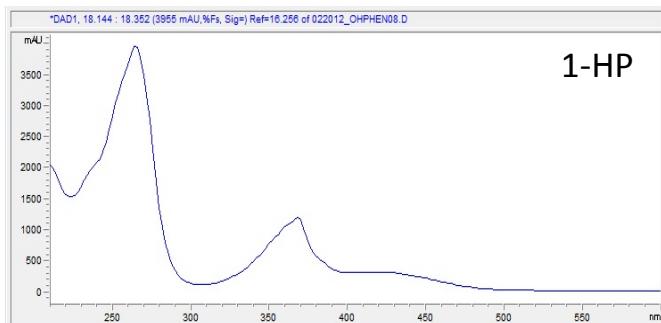


Figure S9.1. (A) HPLC chromatograms (254 nm, 0-22 min time frame) of nBuOH extracts from *A. fumigatus* (24 h pregrown) – phenazine incubations (ISP2, 30°C, 24h). a) PCN; b) 1-MP; c) 1-HP; d) PCA; e) PYO. (B) UV chromatographs of 1-HP, 1-MP, PCA and phenazine-1-sulfate. HPLC gradient: 10 to 100% ACN in H₂O with 0.1% TFA (35 min), hold (3 min), back to starting conditions (2 min).

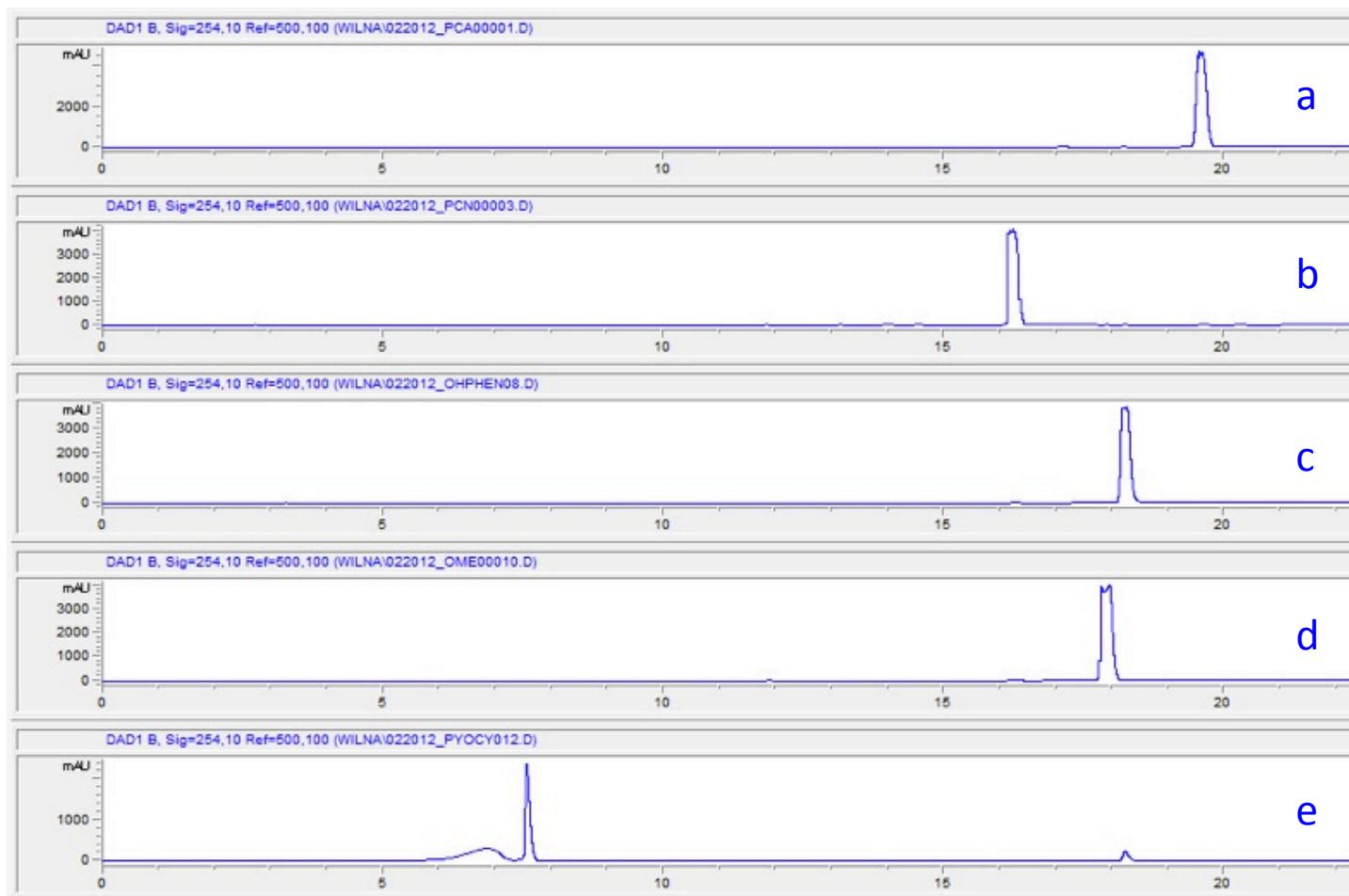


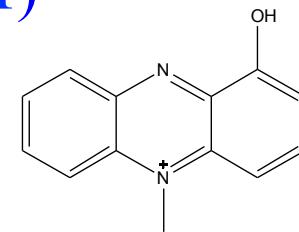
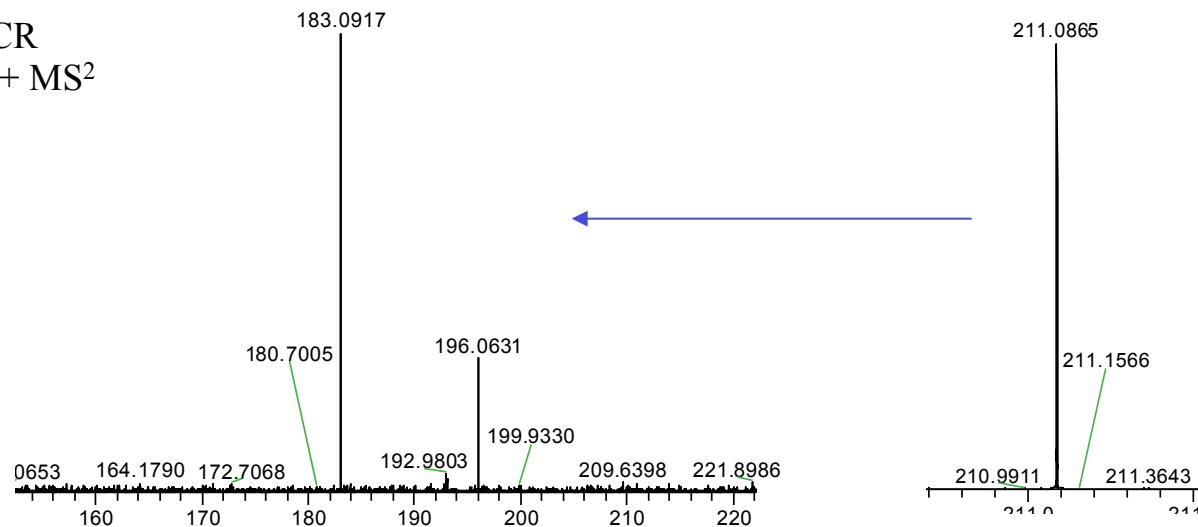
Figure S9.2. Corresponding HPLC chromatograms (254 nm, 0-22 min time frame) of different standard phenazines. a) PCA; b) PCN; c) 1-HP; d) 1-MP; e) PYO.
HPLC gradient: 10 to 100% ACN in H₂O with 0.1% TFA (35 min), hold (3 min), back to starting conditions (2 min).

C) Spectral analyses

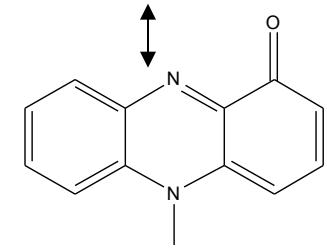
Pyocyanin (PYO; compound 1)

Commercial from Sigma-Aldrich

FT-ICR
MS¹ + MS²

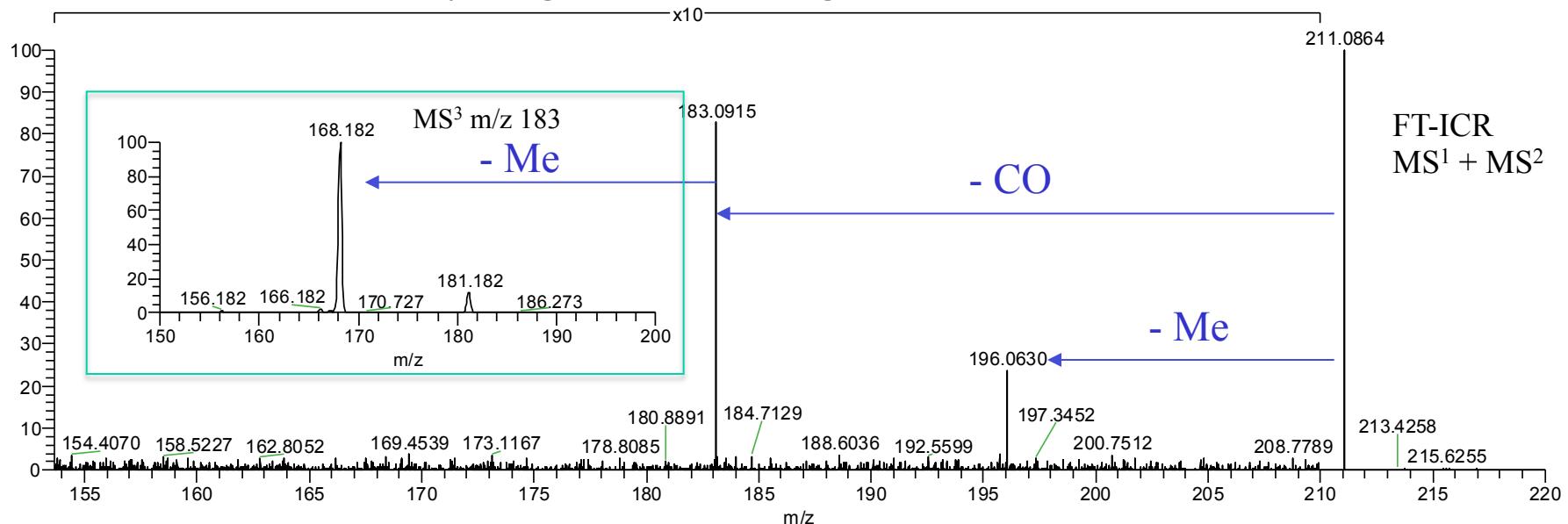


Chemical Formula: C₁₃H₁₁N₂O⁺
Exact Mass: 211.09



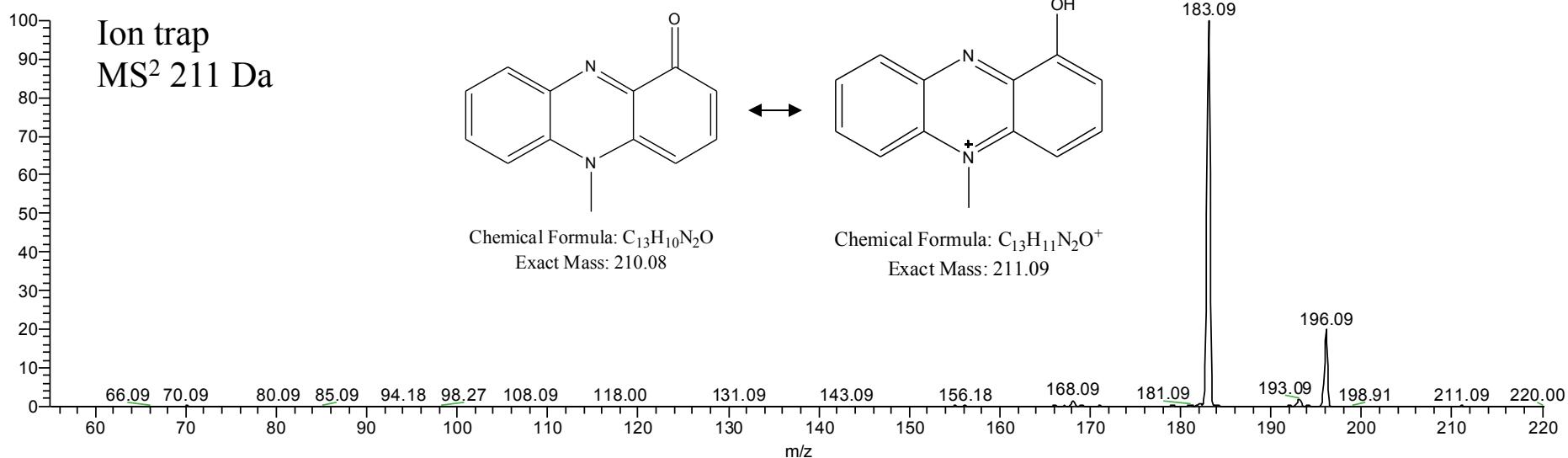
Calc [M+H]⁺ = 211.0866
Obs [M+H]⁺ = 211.0864
Δ = -0.9 ppm

EtOAc extract from *A. fumigatus* – *P. aeruginosa* interaction

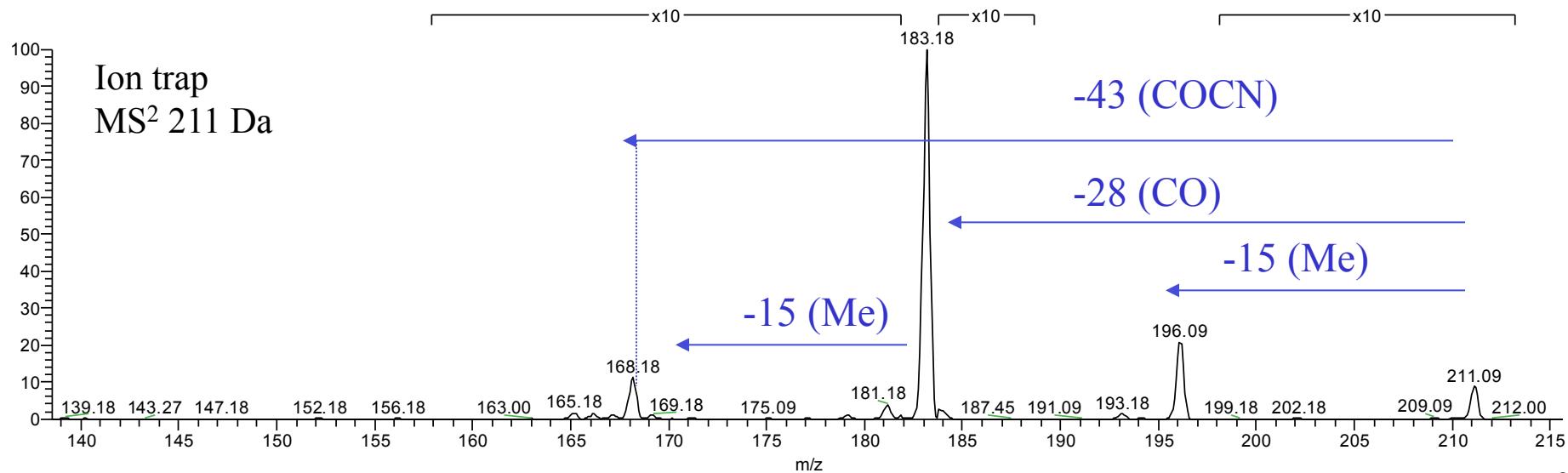


PYO (compound 1)

Commercial from Sigma-Aldrich



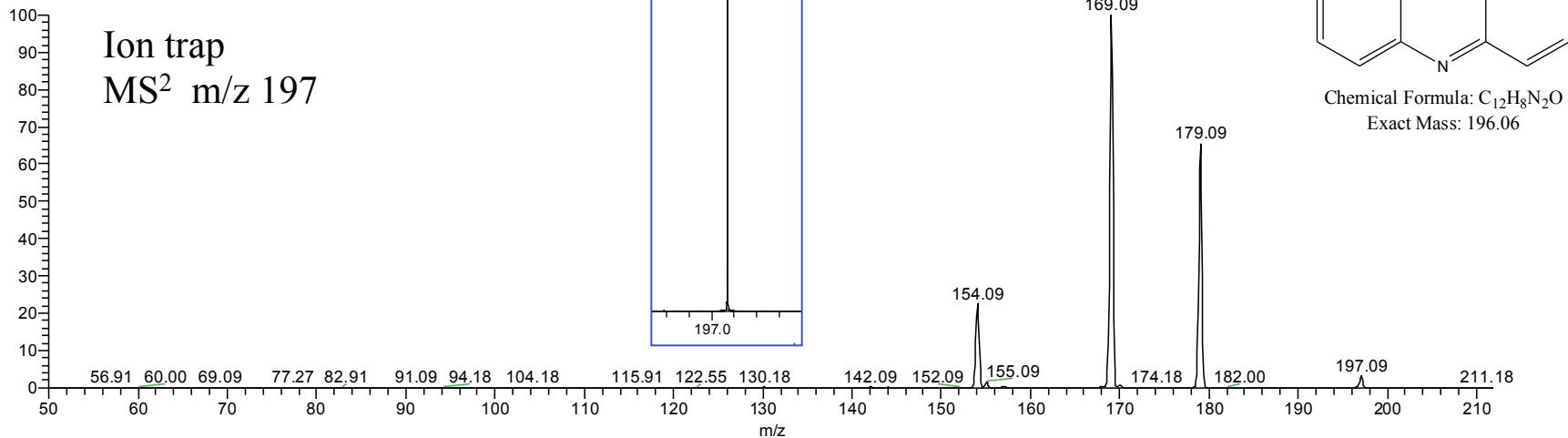
EtOAc extract from *A. fumigatus* - *P. aeruginosa* interaction



1-Hydroxyphenazine (1-HP; compound 2)

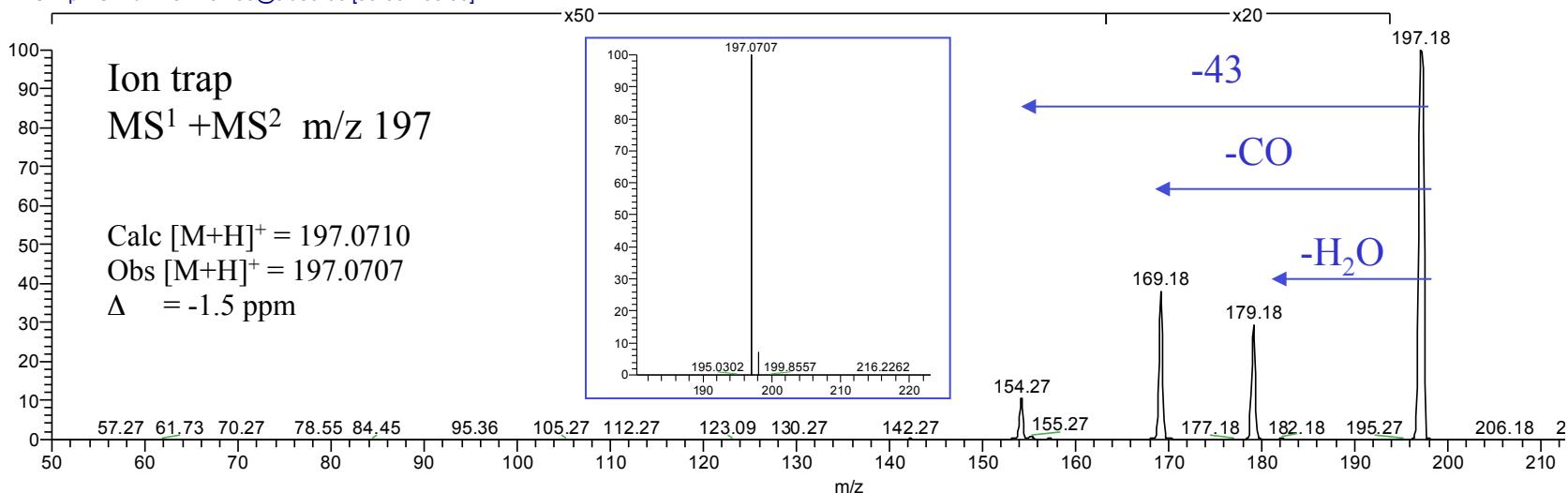
Commercial from TCI

Hydroxyphenazine_IT-197_35 #1-44 R⁻ 0.81 AV: 44 NL: 8.95E2
T: ITMS + p NSI Full ms2 197.20@cid35.00 [50.00-1000.00]



EtOAc extract from *A. fumigatus* - *P. aeruginosa* interaction

090411AfumPA14_3_140_fr6_IT197 #1-ε : 0.00-1.87 AV: 586 NL: 1.87E4
T: ITMS + p NSI Full ms2 197.00@cid35.00 [50.00-400.00]

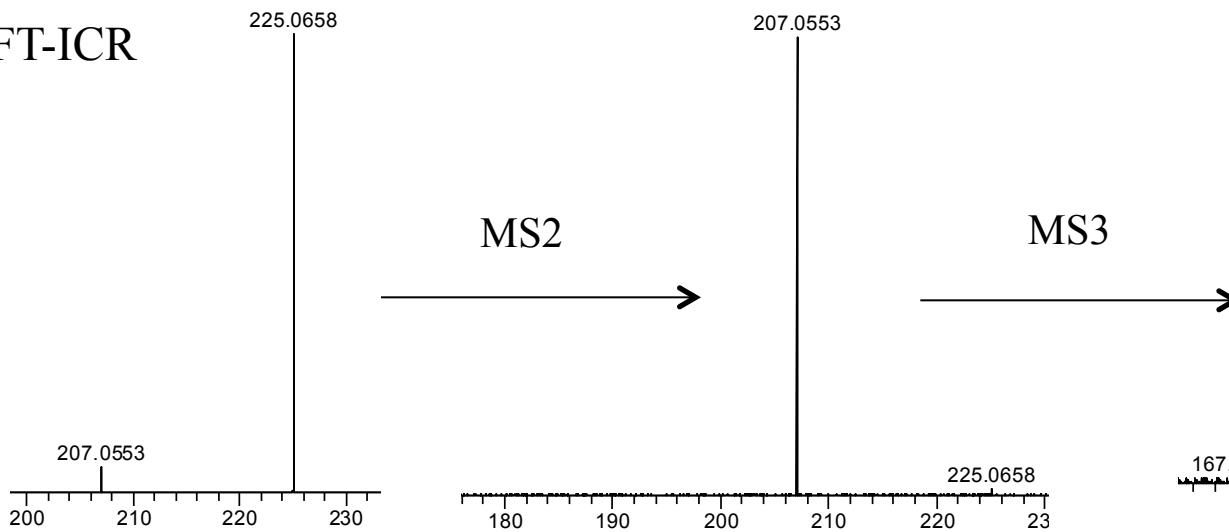


Fragmentation data in accordance with previous reports.⁷

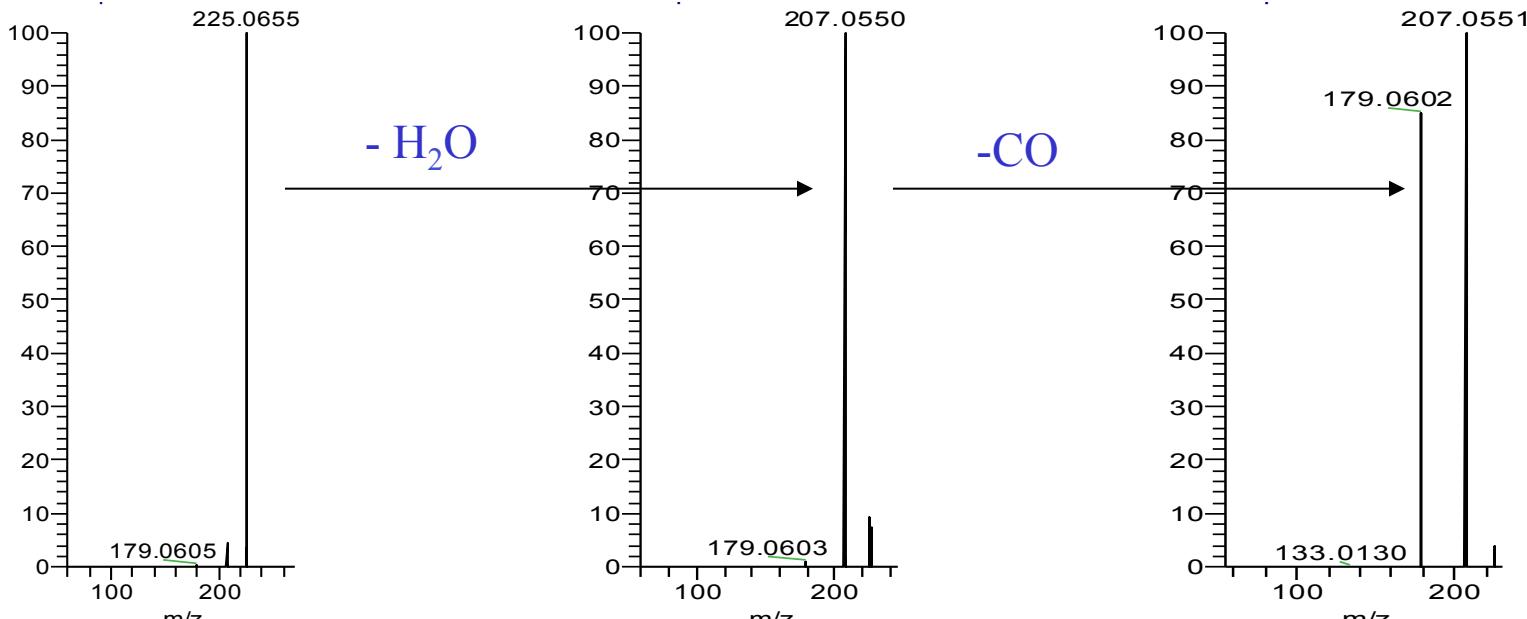
Phenazine-1-carboxylic acid (PCA; compound 3)

Commercial from Princeton Bioblocks

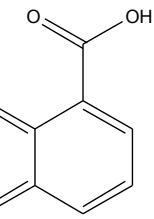
FT-ICR



EtOAc extract *A. fumigatus* - *P. aeruginosa* interaction



Fragmentation data in accordance with previous reports.⁷



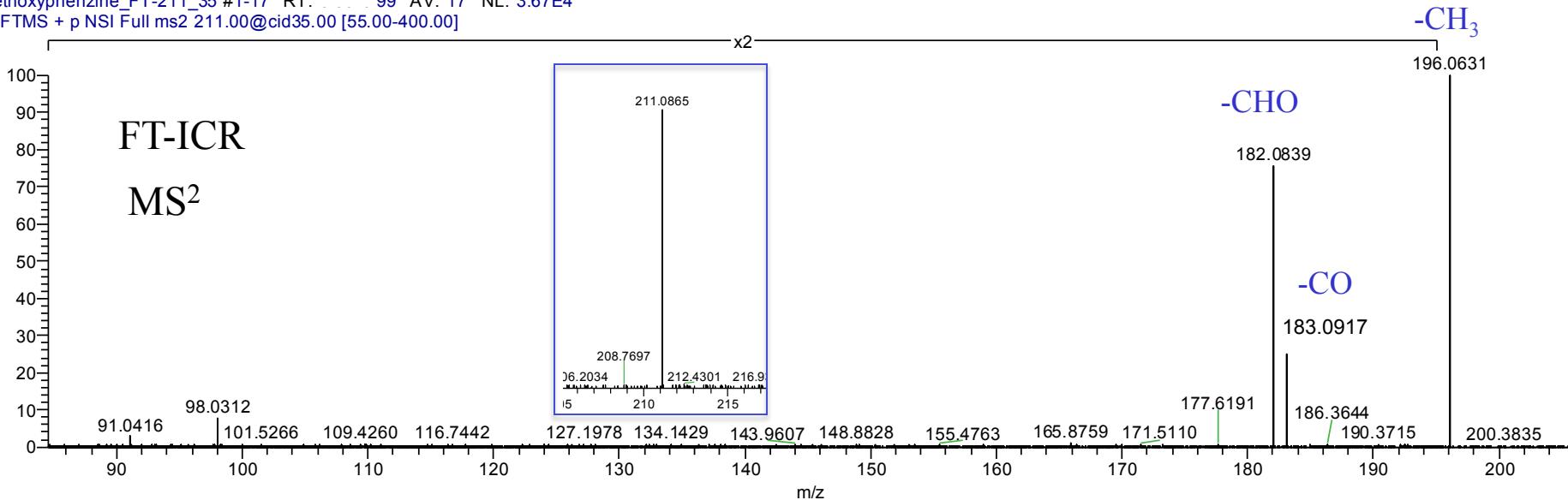
Chemical Formula: C₁₃H₈N₂O₂
Exact Mass: 224.06

Calc [M+H]⁺ = 225.0656
Obs [M+H]⁺ = 225.0655
Δ = -0.4 ppm

1-Methoxyphenazine (1-MP; compound 4)

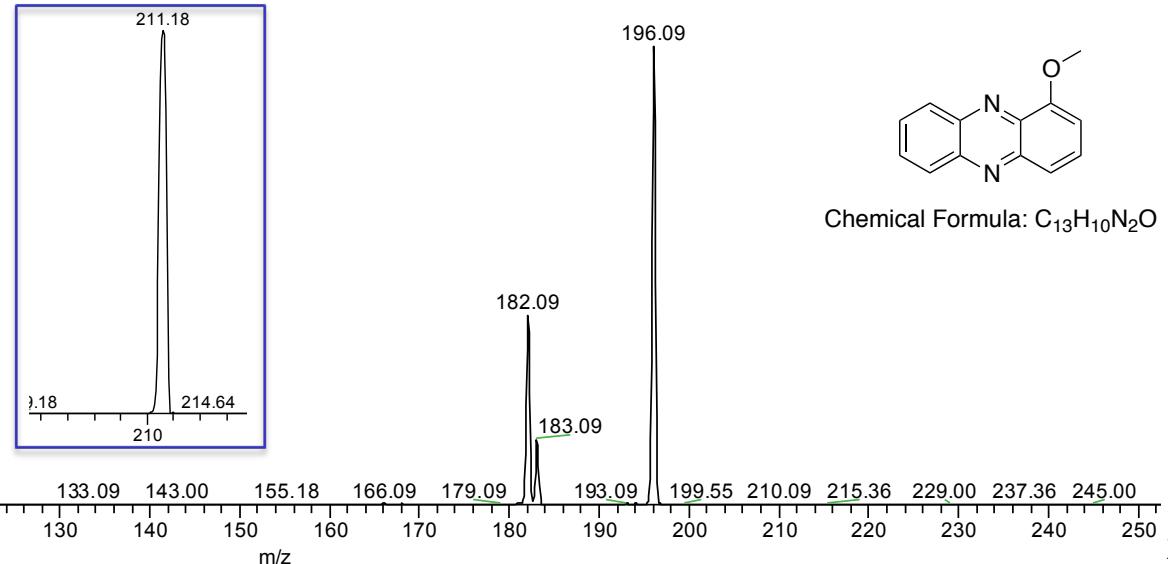
Commercial from TCI

methoxyphenazine_FT-211_35 #1-17 RT: 99 AV: 17 NL: 3.67E4
T: FTMS + p NSI Full ms2 211.00@cid35.00 [55.00-400.00]



methoxyphenazine_IT-211-35 #1-53 RT: .95 AV: 53 NL: 1.39E4
T: ITMS + p NSI Full ms2 211.00@cid35.00 [55.00-1000.00]

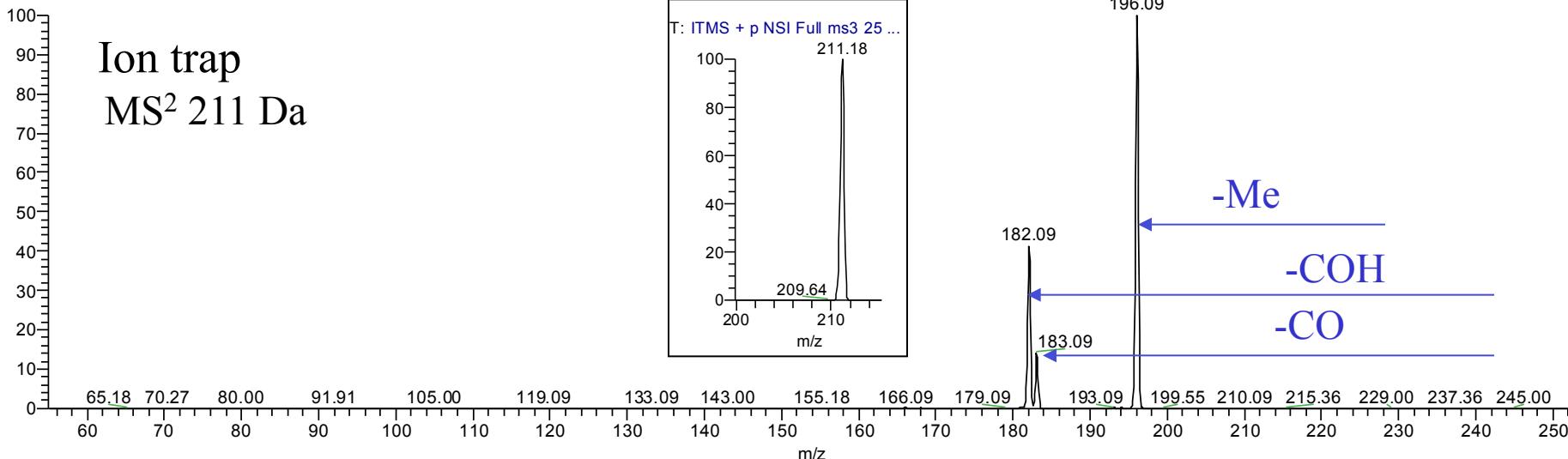
Ion Trap
MS²



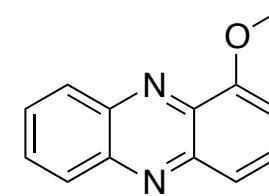
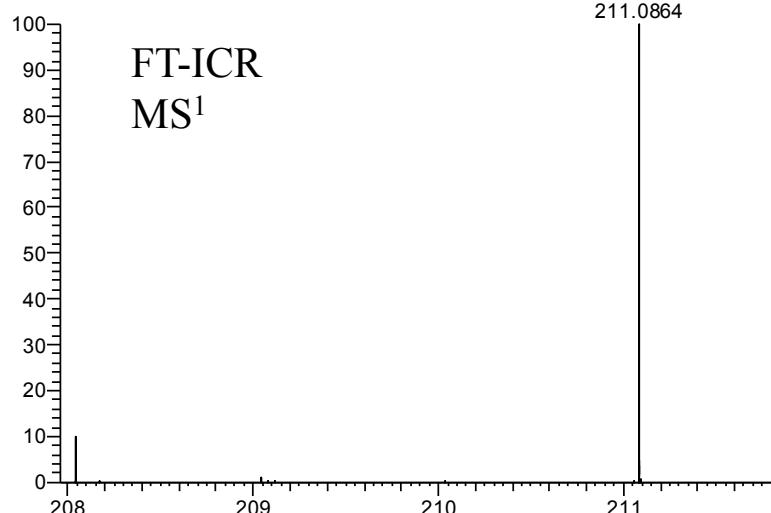
1-MP (compound 4)

EtOAc extract from *A. fumigatus* – *P. aeruginosa* interaction

methoxyphenazine_IT-211-35 #1-53 RT: 11.95 AV: 53 NL: 1.39E4
T: ITMS + p NSI Full ms2 211.00@cid35.00 [55.00-1000.00]



AfumPA14Extract140-10_FT #1-50 RT: 11.80 AV: 50 NL: 3.29E5
T: FTMS + p NSI Full ms [50.00-1000.00]



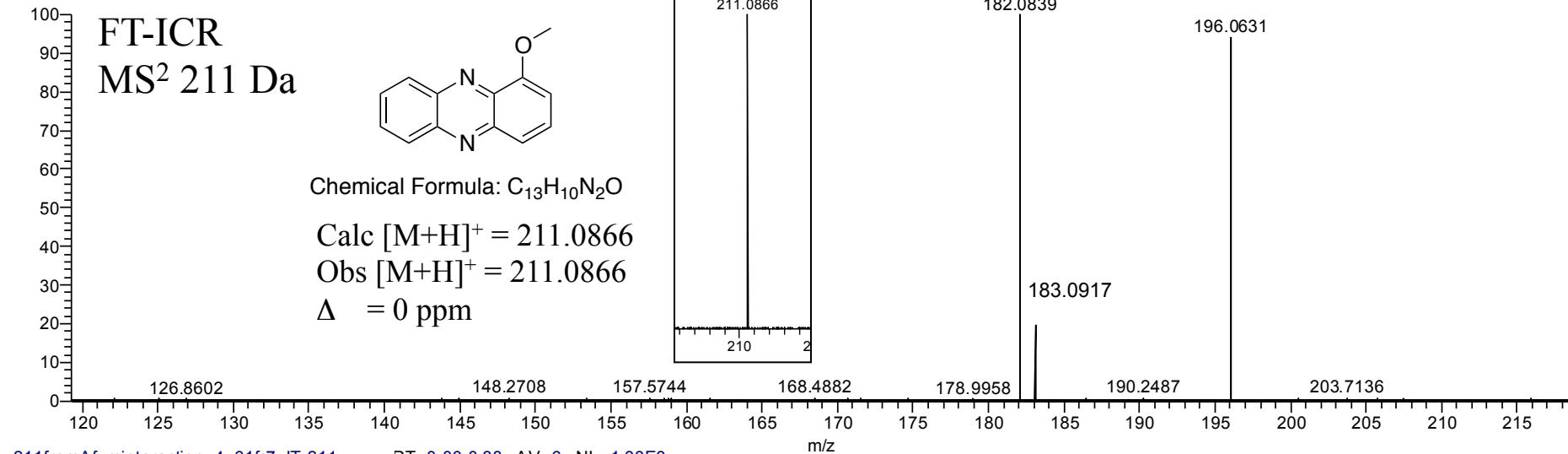
Chemical Formula: C₁₃H₁₀N₂O

Calc [M+H]⁺ = 211.0866
Obs [M+H]⁺ = 211.0864
Δ = -0.9 ppm

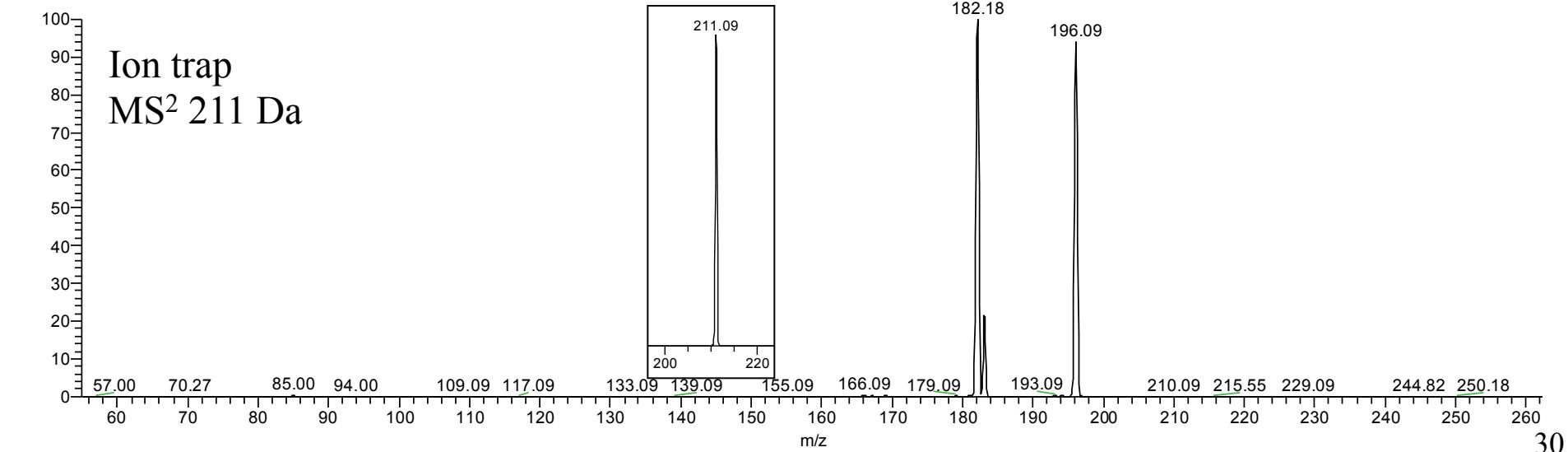
1-MP (compound 4)

Metabolism product from 1-hydroxyphenazine by *A. fumigatus* (EtOAc extract)

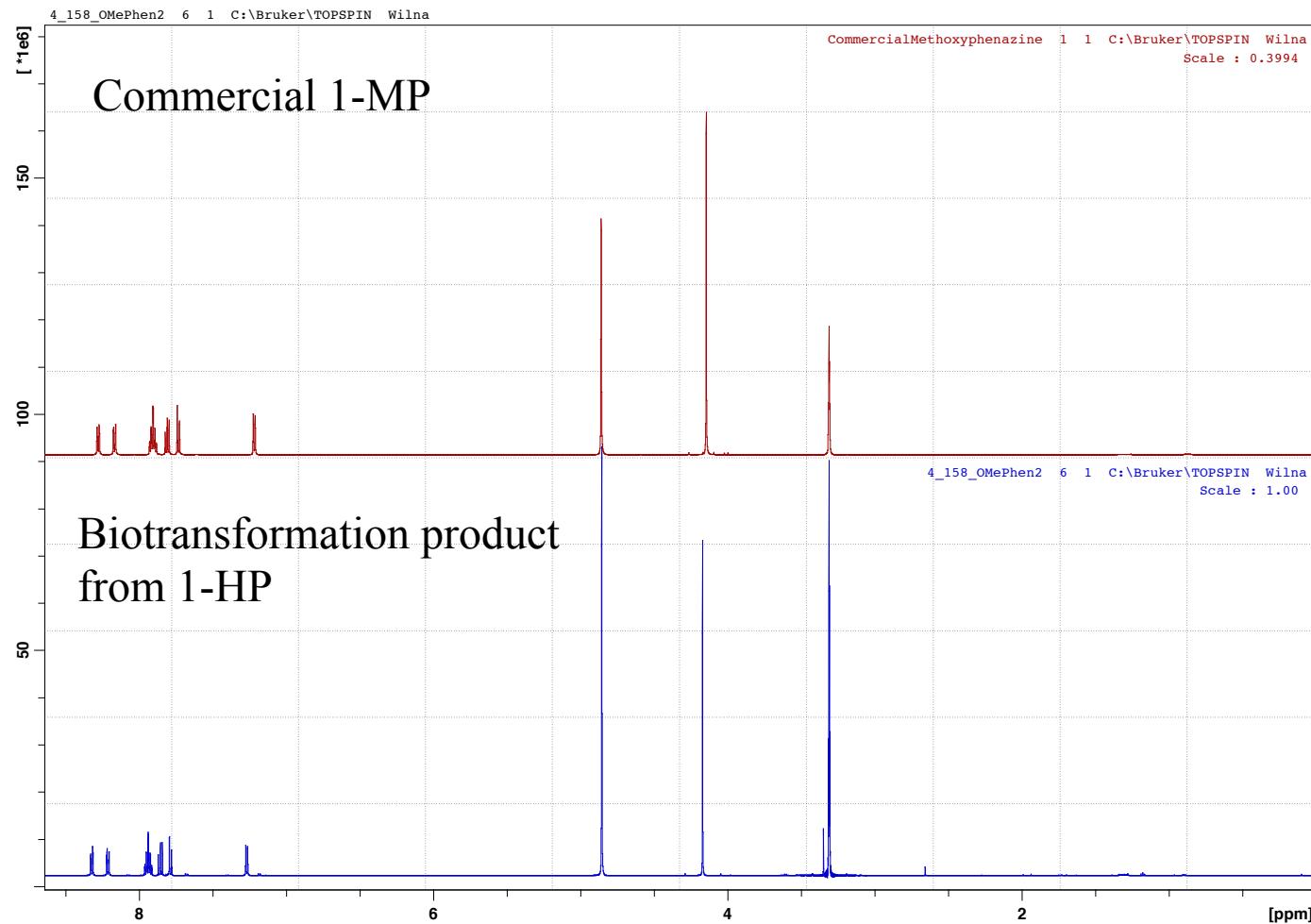
211fromAfuminteraction_4_61fr6_Ft_21 32 RT: 1.34-4.01 AV: 106 NL: 2.62E3
T: FTMS + p NSI Full ms2 211.00@cid35.00 [55.00-1000.00]



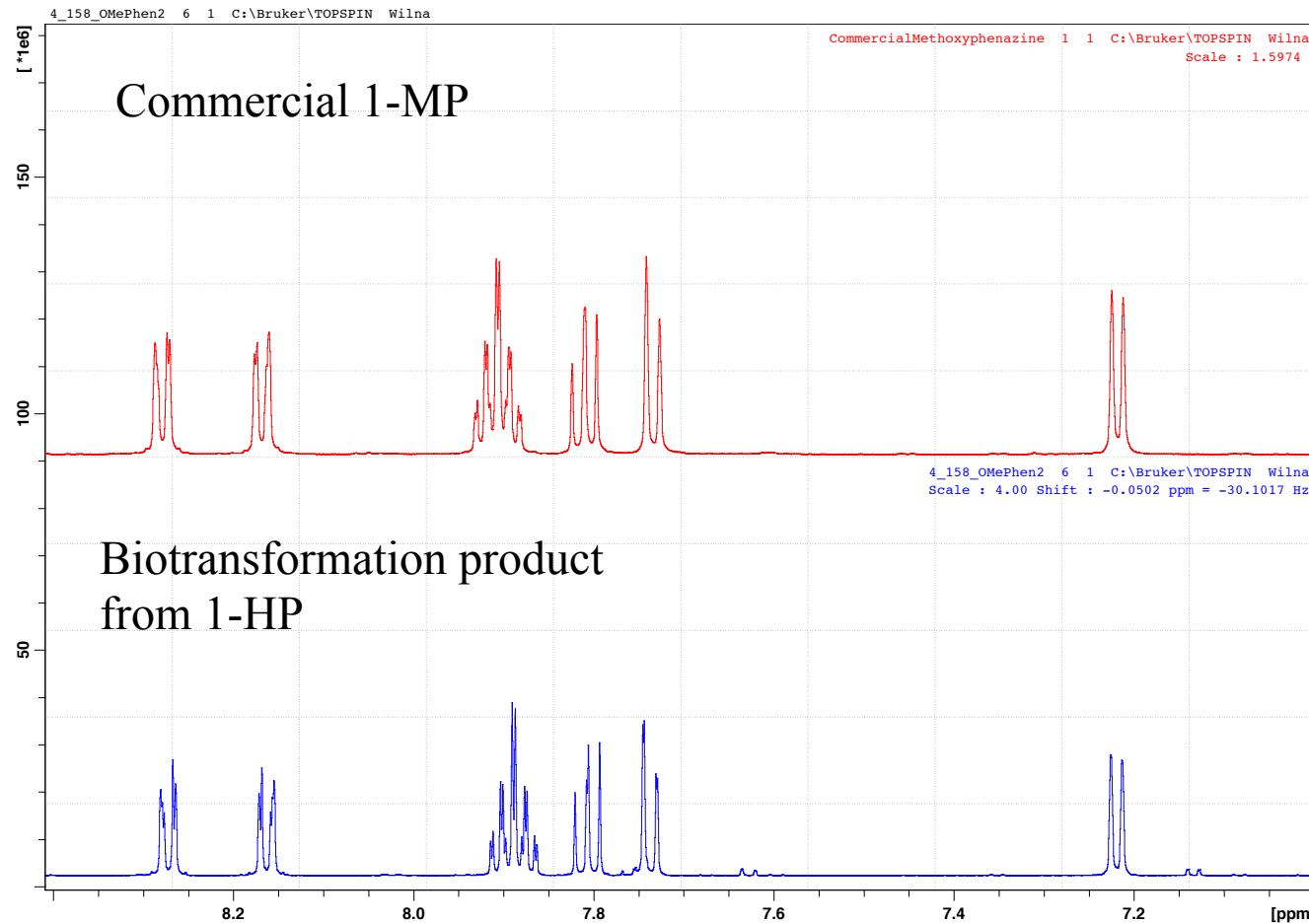
211fromAfuminteraction_4_61fr7_IT-211 RT: 0.00-0.38 AV: 6 NL: 1.33E3
T: ITMS + p NSI Full ms2 211.00@cid35.00 [55.00-1000.00]



¹H NMR of commercial 1-MP compared with metabolite product from 1-HP by *A. fumigatus*



¹H NMR of commercial 1-MP compared with metabolite product from 1-HP by *A. fumigatus*



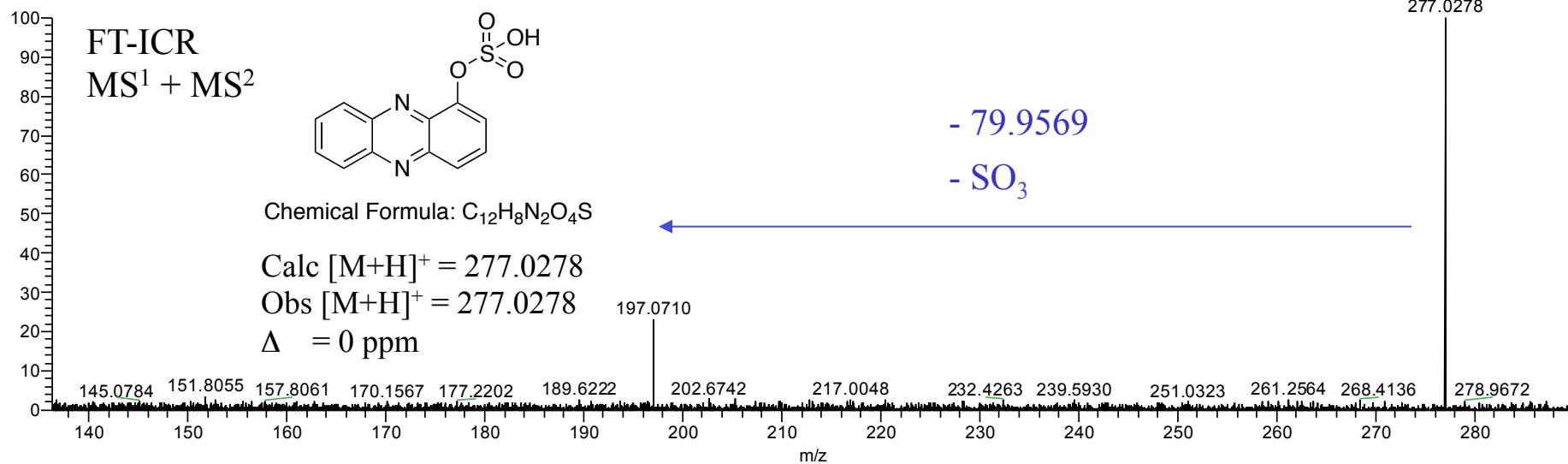
¹H NMR (MeOD, 600 MHz) 4.17 (s, 3H), 7.27 (d, 1H, $J = 7.5$ Hz), 7.79 (dd, 1H, $J = 8.8$ Hz, $J = 0.96$ Hz), 7.86 (dd, 1H, $J = 7.5$ Hz, $J = 8.8$ Hz), 7.91-7.96 (m, 2H), 8.20-8.22 (m, 1H), 8.31-8.33 (m, 1H).

Phenazine-1-sulfate (compound 5)

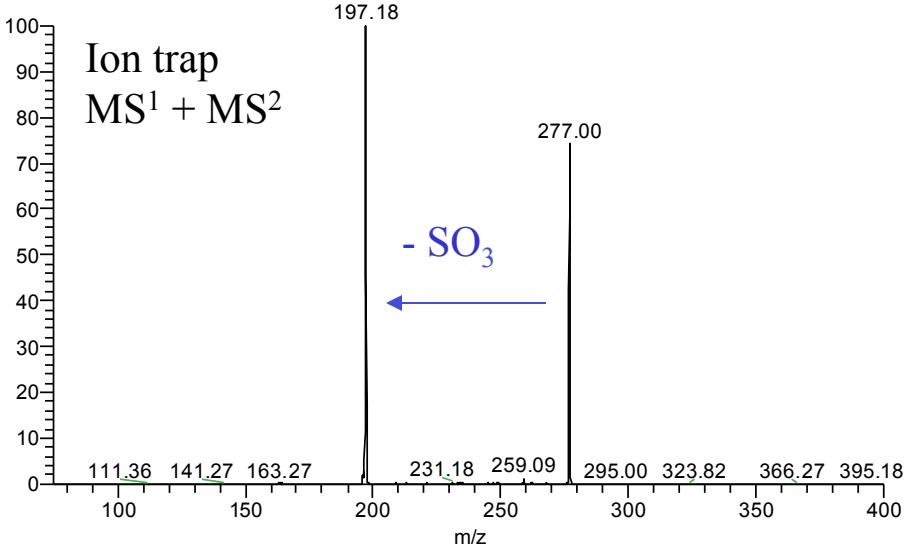
MeOH extract isolated from *A. fumigatus* - *P. aeruginosa* interaction.

Same fragmentation for m/z 277 Da isolated from interaction of *A. fumigatus* with 1-HP and PCA

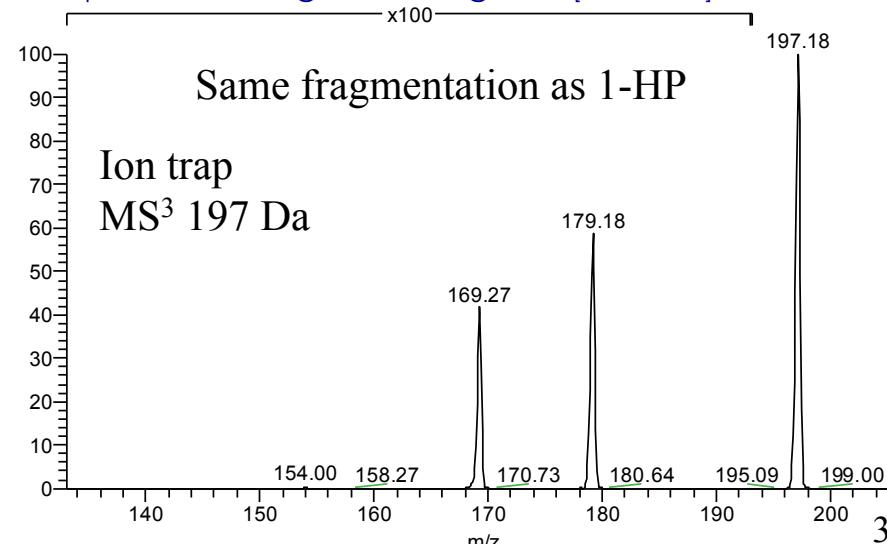
090411AfumPA14_4_6Fr7_FT277 #1-28 0.00-2.59 AV: 28 NL: 6.73E2
T: FTMS + p NSI Full ms2 277.00@cid0.00 [75.00-400.00]



090411AfumPA14_4_6Fr7_IT277 #1-11 0.00-0.84 AV: 110 NL: 7.24E2
T: ITMS + p NSI Full ms2 277.00@cid35.00 [75.00-400.00]

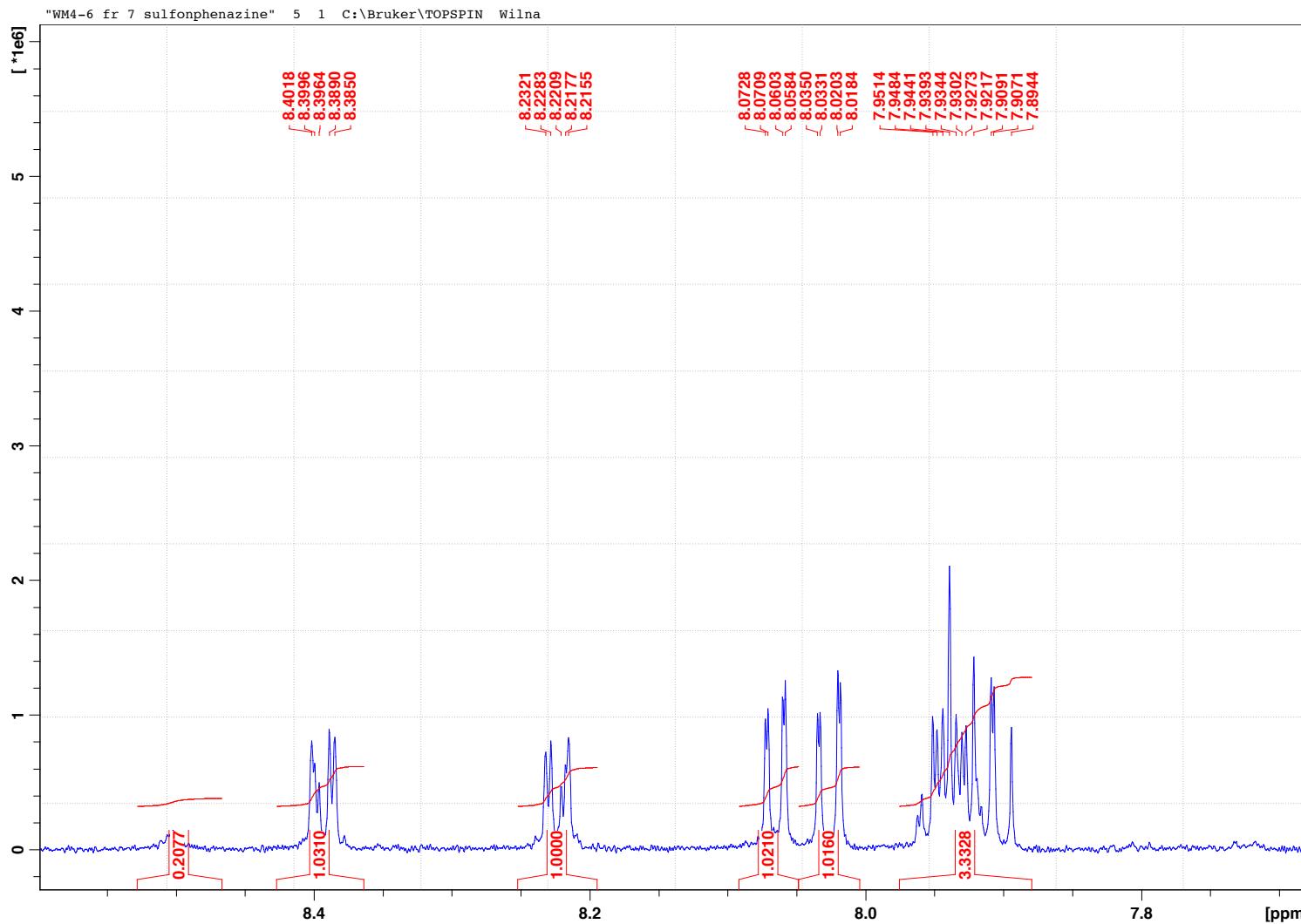


090411AfumPA14_4_6Fr7_IT277_197 #2 0.01-1.69 AV: 210 NL: 2.05E2
T: ITMS + p NSI Full ms3 277.00 197.00@cid35.00 [50.00-400.00]

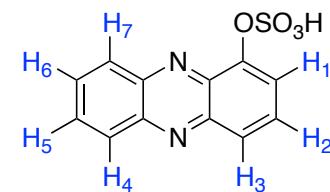
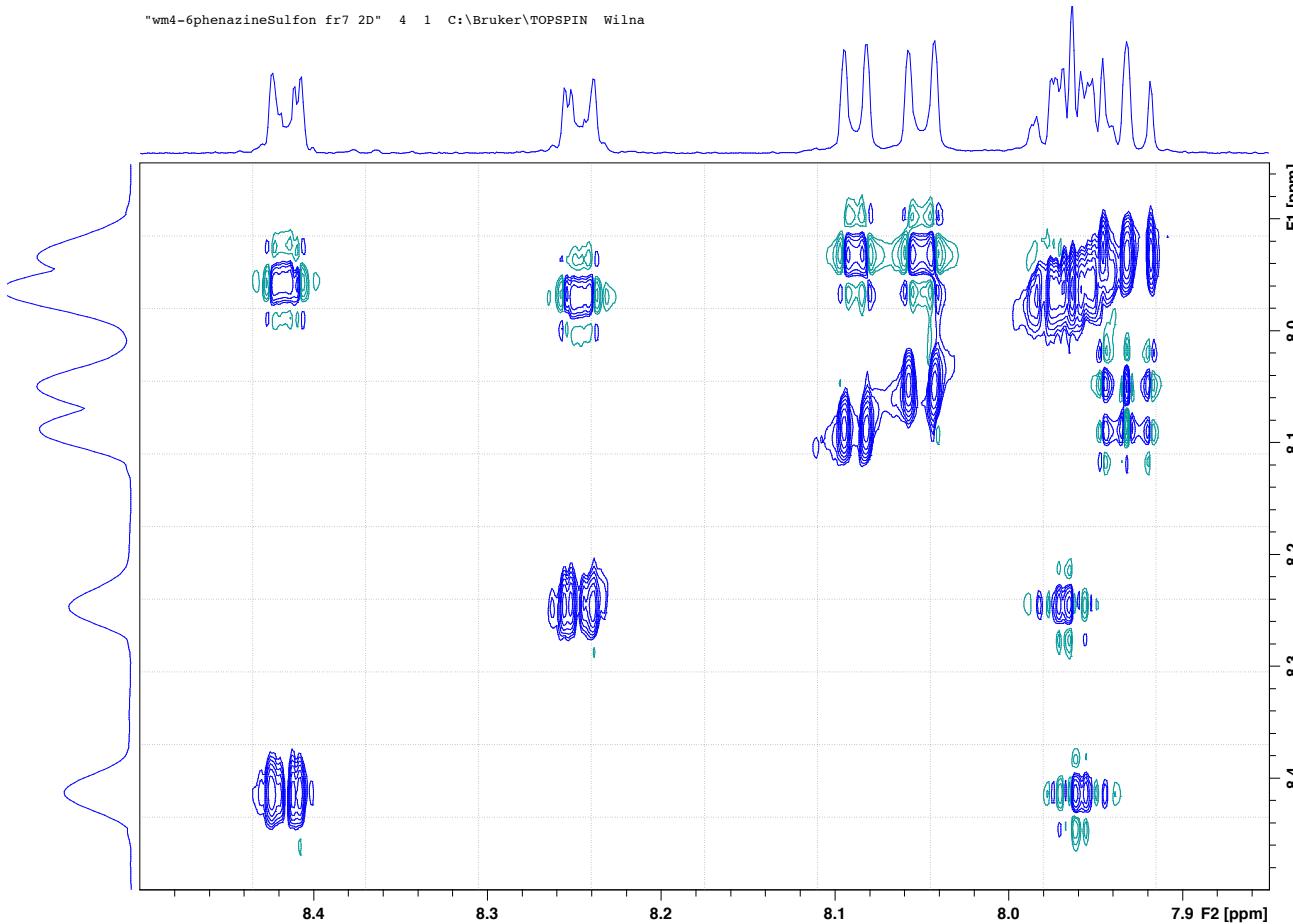


^1H NMR of phenazine-1-sulfate (compound 5)

MeOH extract of interaction *A. fumigatus* – *P. aeruginosa*



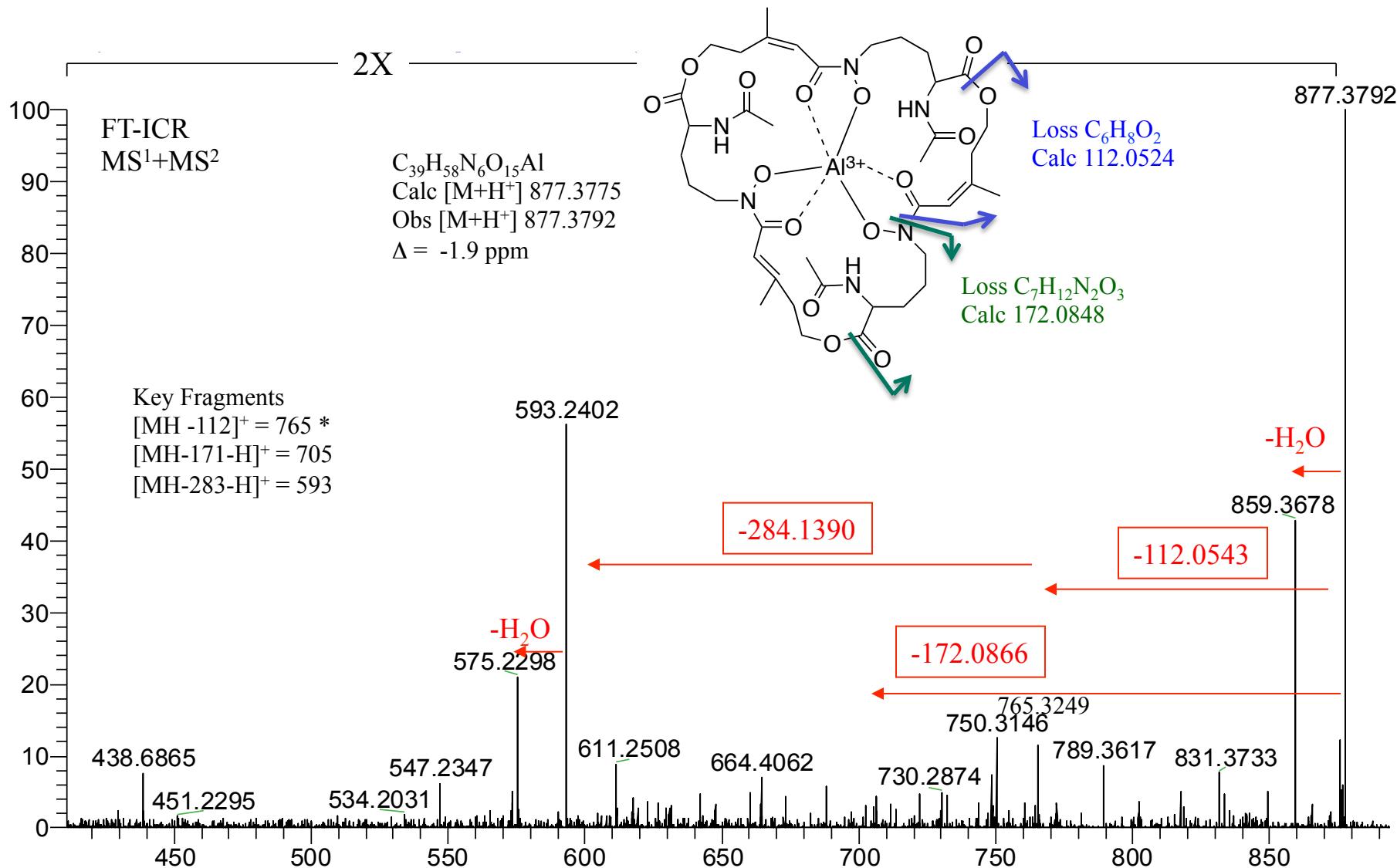
COSY of phenazine-1-sulfate (compound 5)



¹H NMR (MeOD, 600 MHz) 7.91 (**H₂**, dd, 1H, *J* = 7.6 Hz, *J* = 8.8 Hz), 7.92-7.97 (**H₅**, **H₆**, m, 2H), 8.03 (**H₁** or **H₃**, dd, 1H, *J* = 8.8 Hz, *J* = 1.1 Hz), 8.06 (**H₁** or **H₃**, dd, 1H, *J* = 7.5 Hz, *J* = 1.1 Hz), 8.38-8.40 (**H₄** or **H₇**, m, 1H), 8.31-8.33 (**H₄** or **H₇**, m, 1H).

Al^{3+} complexed triacetyl fusarinine C (compound 6)

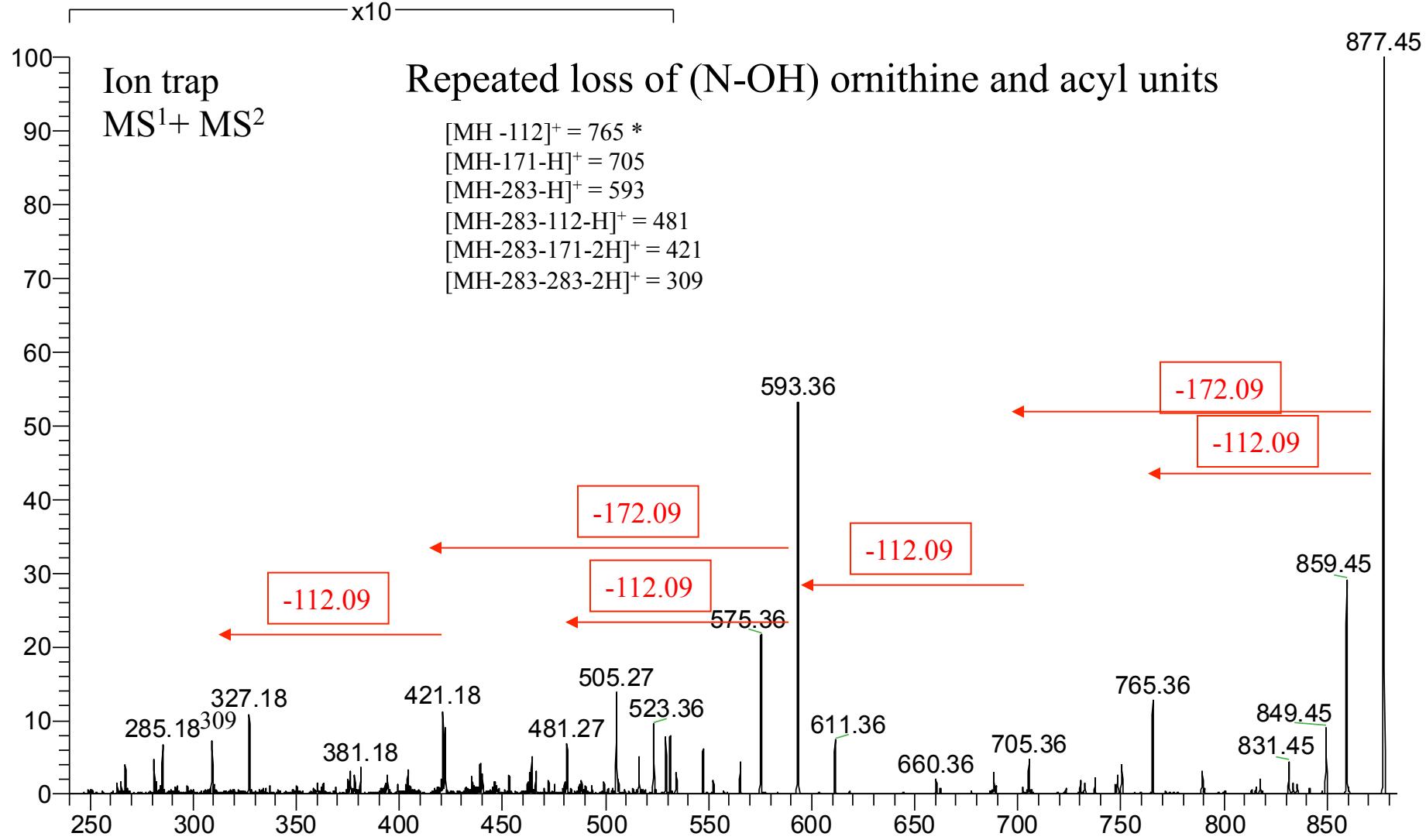
EtOAc extract from *A. fumigatus* – *P. aeruginosa* interaction
Same data for EtOAc extract *A. fumigatus* /1-HP interaction



Al^{3+} complexed triacetyl fusarinine C (compound 6)

EtOAc extract from *A. fumigatus* – *P. aeruginosa* interaction

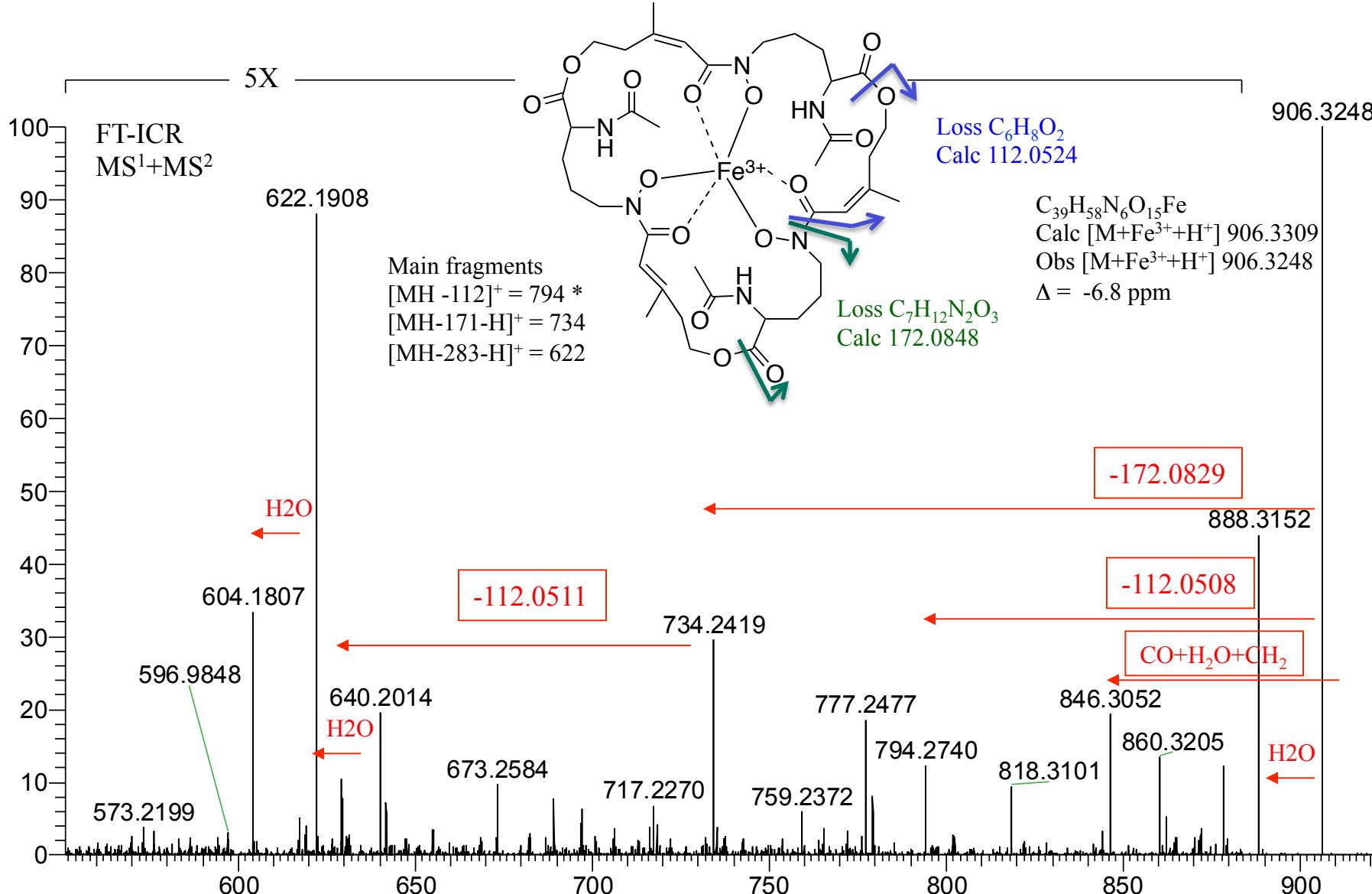
4-6fr8_IT877 #1-54 RT: 0.00-0.64 AV: 54 NL: 3.
T: ITMS + p NSI Full ms2 877.30@cid35.00 [240.00-1000.00]



* Fragmentation analogous to Fe^{3+} bound triacetyl fusarinine C (compound 7) which is in accordance with reported data.^{8,9} 37

Fe³⁺ complexed triacetylfusarinine C (compound 7)

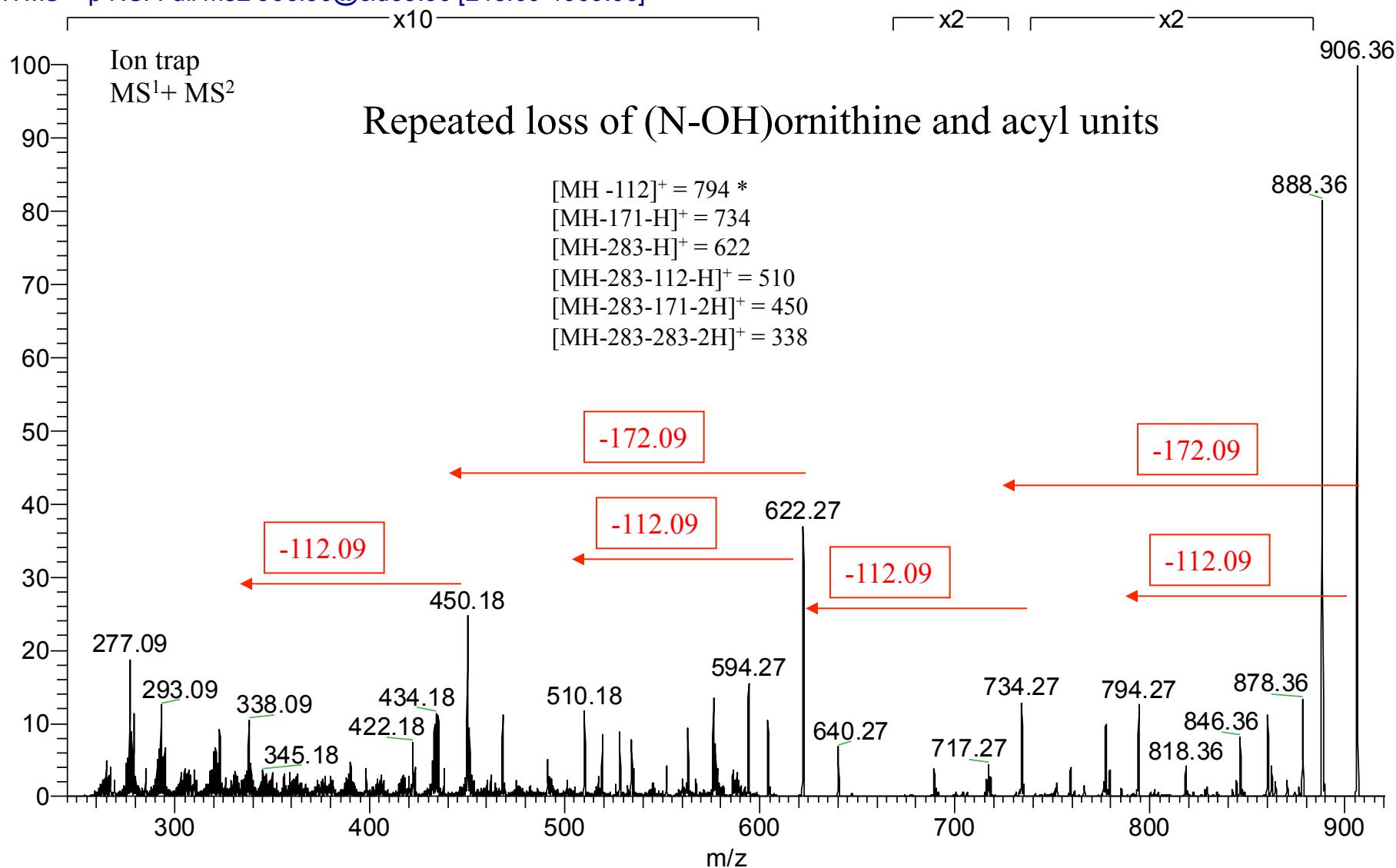
EtOAc extract from *A. fumigatus* – *P. aeruginosa* interaction. Same results for *A. fumigatus* – 1-HP extract



* Fragments are in accordance with reported data.^{8,9}

Fe^{3+} complexed triacetyl fusarinine C (compound 7)

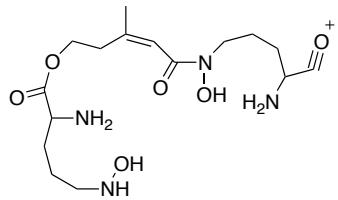
4-6fr8_IT906 #1-100 RT: 0.00-1.57 AV: 100 NL:
T: ITMS + p NSI Full ms2 906.30@cid35.00 [245.00-1000.00]



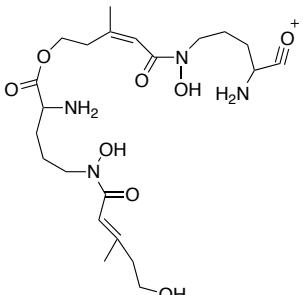
* Fragments are in accordance with reported data.^{8,9}

Fungal siderophore fusarinine C (uncomplexed form of 8)

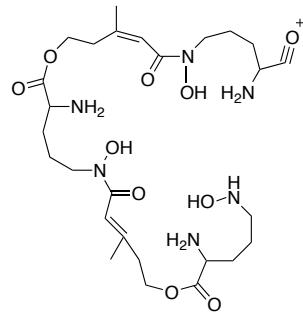
Water/AcN/FA extract from *A. fumigatus* – *P. aeruginosa* interaction



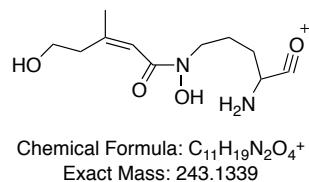
Chemical Formula: $C_{16}H_{29}N_4O_6^+$
Exact Mass: 373.2082



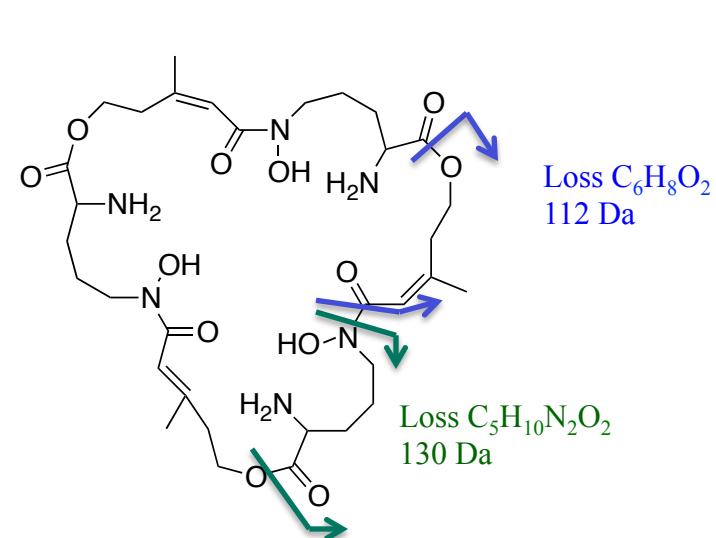
Chemical Formula: $C_{22}H_{37}N_4O_8^+$
Exact Mass: 485.2606



Chemical Formula: $C_{27}H_{47}N_6O_{10}^+$
Exact Mass: 615.3348



Chemical Formula: $C_{11}H_{19}N_2O_4^+$
Exact Mass: 243.1339



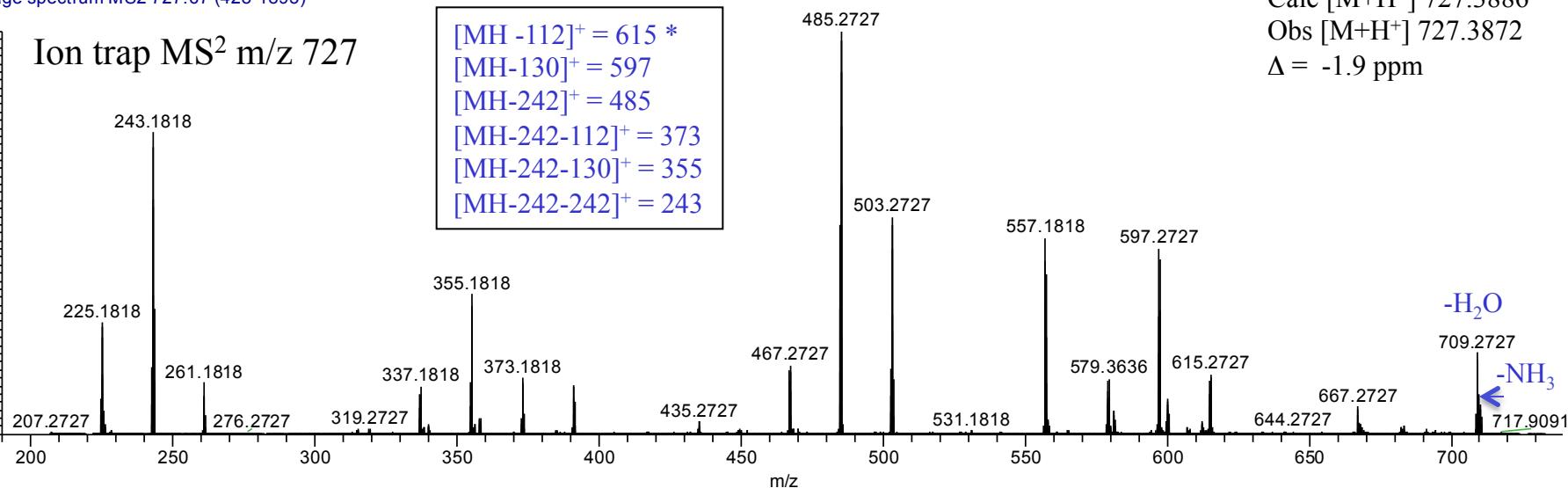
Loss $C_6H_8O_2$
112 Da

Loss $C_5H_{10}N_2O_2$
130 Da

120208_PA14_Af293_1 #428-1895 RT 9.62 AV: 9 NL: 2.75E2
T: Average spectrum MS2 727.67 (428-1895)

Ion trap MS² m/z 727

$[MH - 112]^+ = 615^*$
 $[MH - 130]^+ = 597$
 $[MH - 242]^+ = 485$
 $[MH - 242 - 112]^+ = 373$
 $[MH - 242 - 130]^+ = 355$
 $[MH - 242 - 242]^+ = 243$

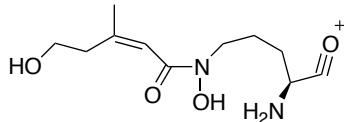


$C_{33}H_{54}N_6O_{12}$
Calc [M+H⁺] 727.3886
Obs [M+H⁺] 727.3872
 $\Delta = -1.9$ ppm

Aluminium complexed siderophore fusarinine C (compound 8)

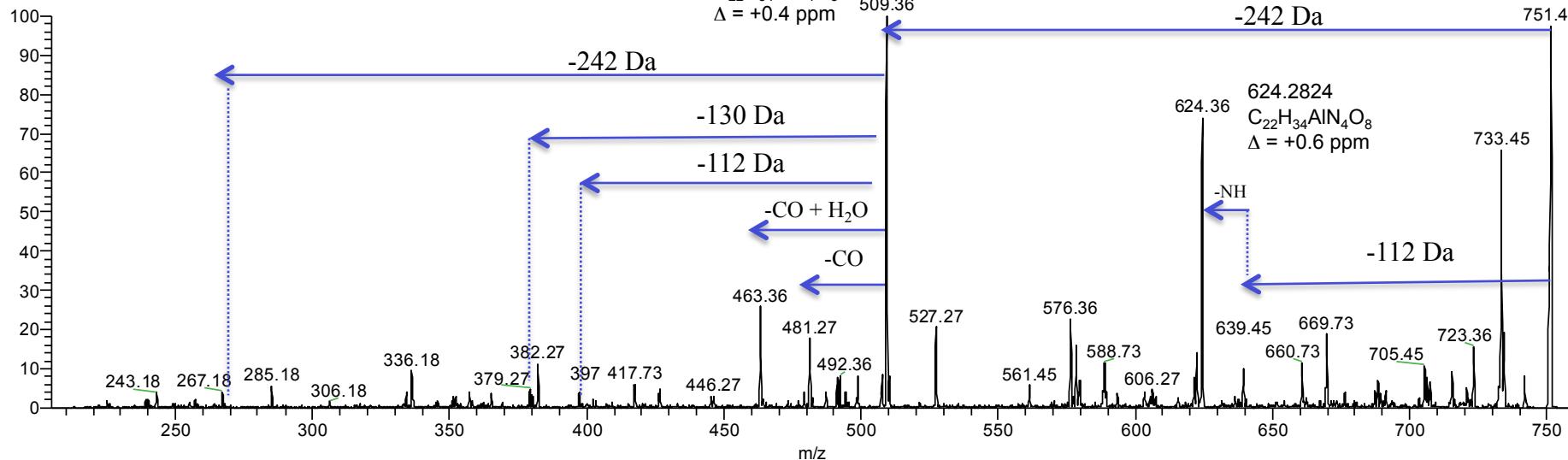
Water/FA extract from *A. fumigatus* – *P. aeruginosa* interaction

Ion trap
MS¹+MS²



Chemical Formula: $C_{11}H_{19}N_2O_4^+$
Exact Mass: 243.1339

32012AfumPA14I_AcN_H2O_IT_751 #1 IT: 0.77-3.30 AV: 44 NL: 4.96
T: ITMS + p NSI Full ms2 751.00@cid35.00 [205.00-900.00]

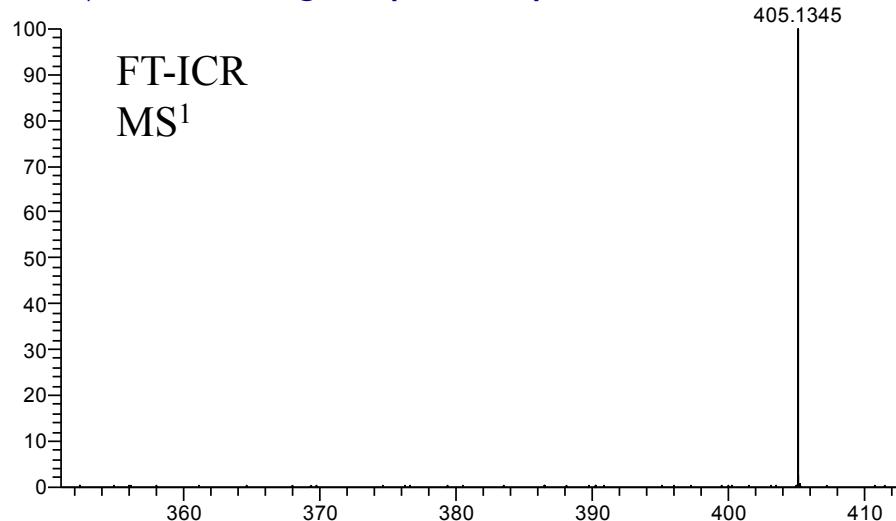


m/z 751 is also observed in purified ferri-fusarinine C but no MS² fragmentation was reported.¹⁰

Phenazine-dimer (compound 9; m/z 405 Da)

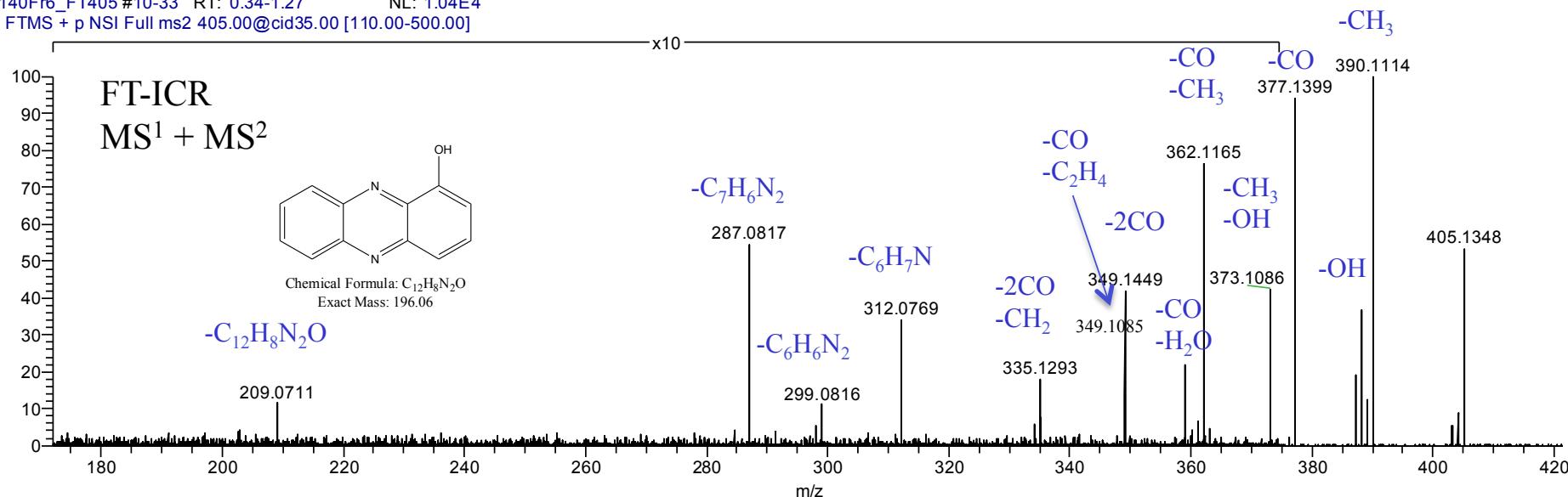
EtOAc extract isolated from *A. fumigatus* - *P. aeruginosa* interaction

3-140Fr6_FT405 #1-9 RT: 0.00-0.30 A IL: 7.29E4
T: FTMS + p NSI Full ms2 405.00@cid0.00 [110.00-500.00]



$C_{25}H_{16}N_4O_2$
Calc $[M+H]^+ = 405.1346$
Obs $[M+H]^+ = 405.1345$
 $\Delta = -0.3$ ppm

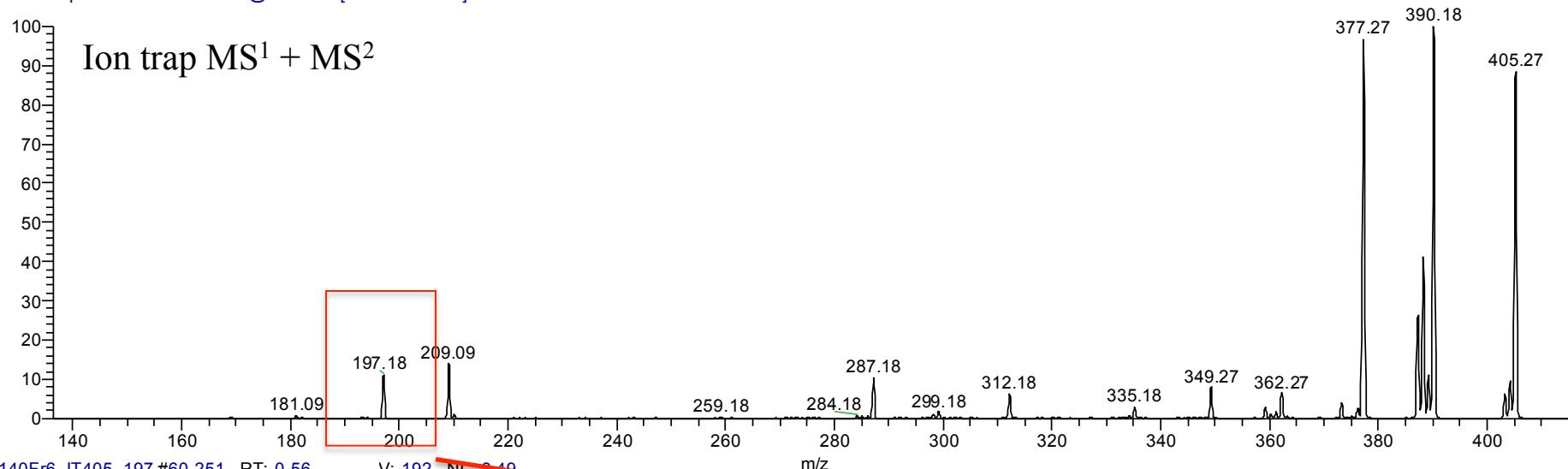
3-140Fr6_FT405 #10-33 RT: 0.34-1.27 NL: 1.04E4
T: FTMS + p NSI Full ms2 405.00@cid35.00 [110.00-500.00]



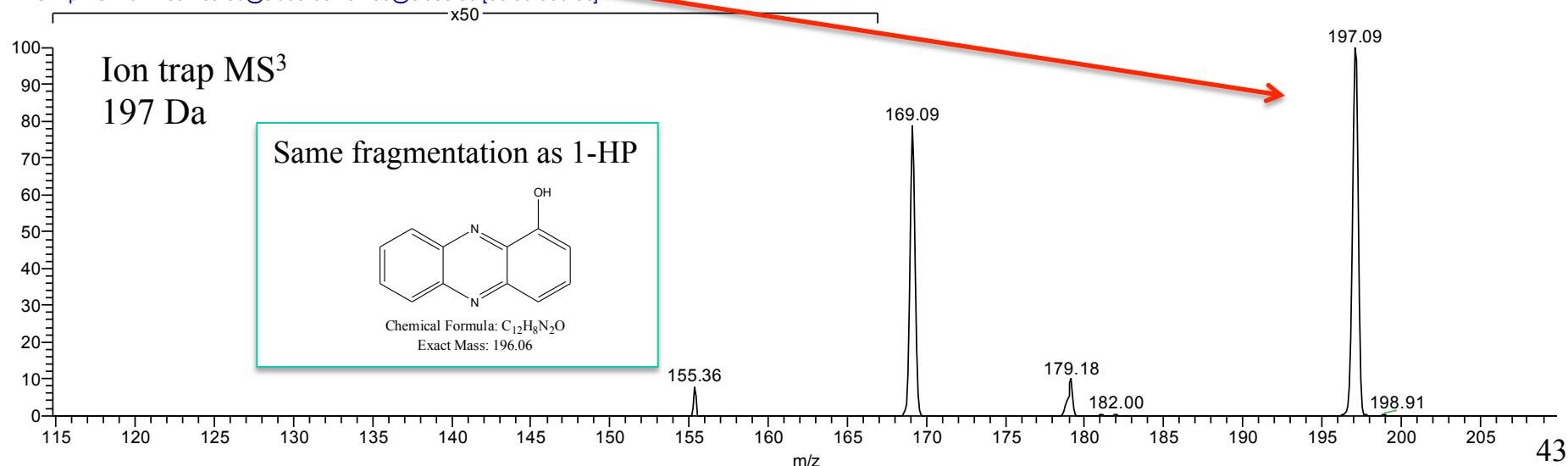
Phenazine-dimer (compound 9; m/z 405 Da)

EtOAc extract isolated from *A. fumigatus* - *P. aeruginosa* interaction

3-140Fr6_IT405_2 #23-109 RT: 0.10-0 ... : 87 NL: 1.50E3
T: ITMS + p NSI Full ms2 405.00@cid35.00 [110.00-500.00]



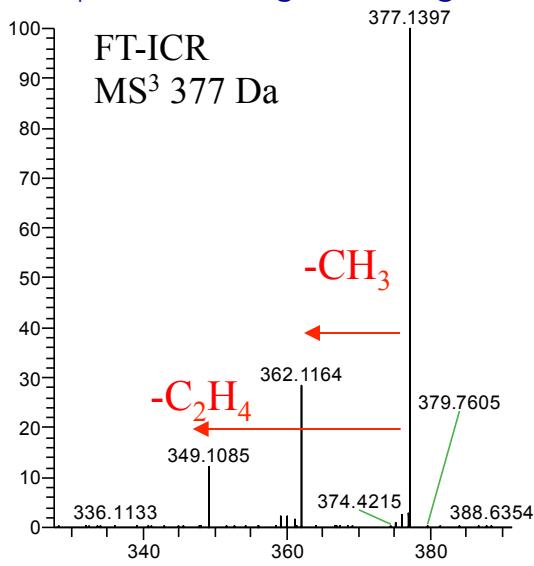
3-140Fr6_IT405_197 #60-251 RT: 0.56- V: 192 NL: 0.40
T: ITMS + p NSI Full ms3 405.00@cid35.00 197.00@cid35.00 [50.00-500.00]



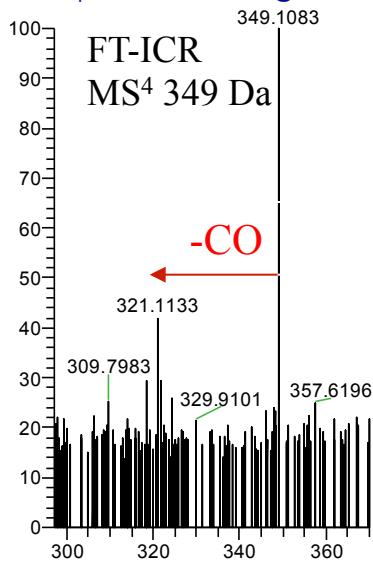
Phenazine-dimer (compound 9; m/z 405 Da)

EtOAc extract isolated from *A. fumigatus* - *P. aeruginosa* interaction

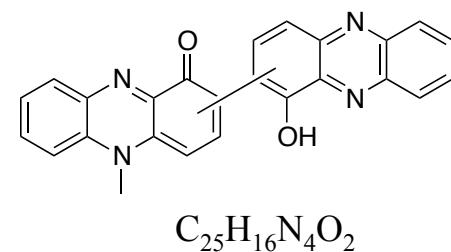
82911PA14_AfumFr6_405_FT_405_377 #1: 0.00-3.05
T: FTMS + p NSI Full ms3 405.00@cid35.00 377.00@cid0.0 ...



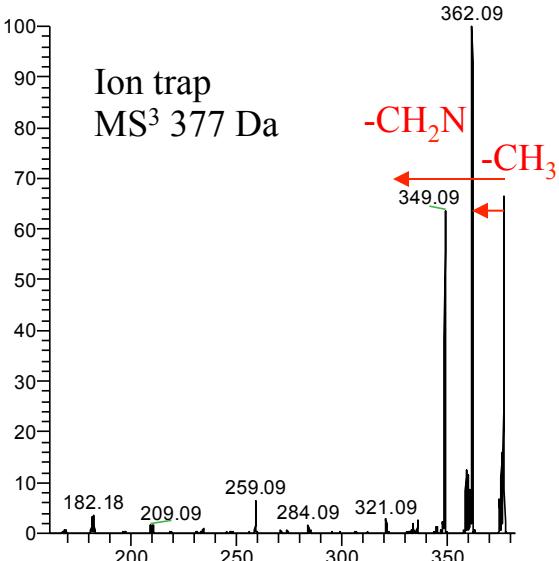
82911PA14_AfumFr6_405_FT_405_377_34
T: FTMS + p NSI Full ms4 405.00@cid35.00 ...



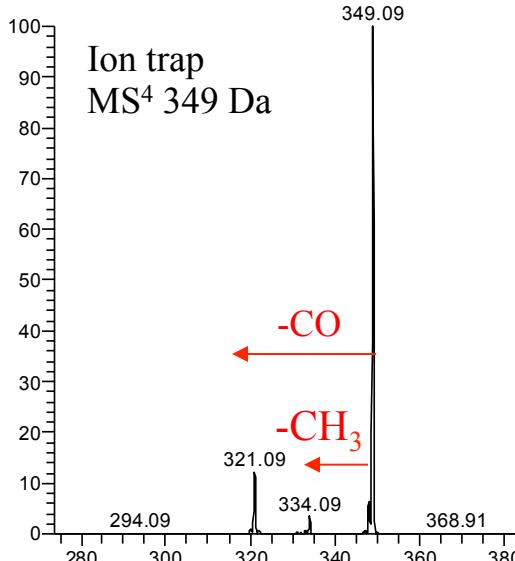
Putative structure



82911PA14_AfumFr6_405_IT_405_377_35 : 0.00 AV:
T: ITMS + p NSI Full ms3 405.00@cid35.00 377.00@cid35.0 ...



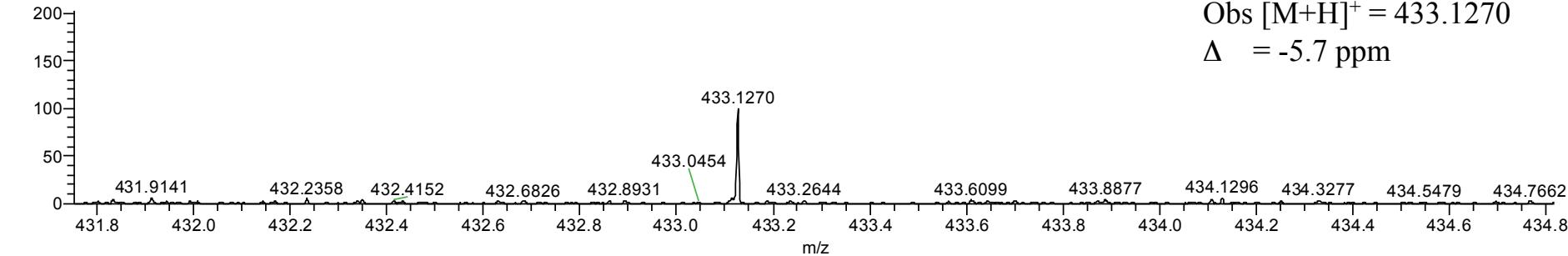
82911PA14_AfumFr6_405_IT_405_377_34!
T: ITMS + p NSI Full ms4 405.00@cid35.00 377.00@cid35.0 ...



Phenazine-dimer (compound 10; m/z 433 Da)

EtOAc extract isolated from *A. fumigatus* - *P. aeruginosa* interaction

11825_PA14_afum_HPLC_111_FTfull #1-111 RT: 0.00-2.06 AV: 111 NL: 5.07E2
T: FTMS + p NSI Full ms [80.00-2000.00]

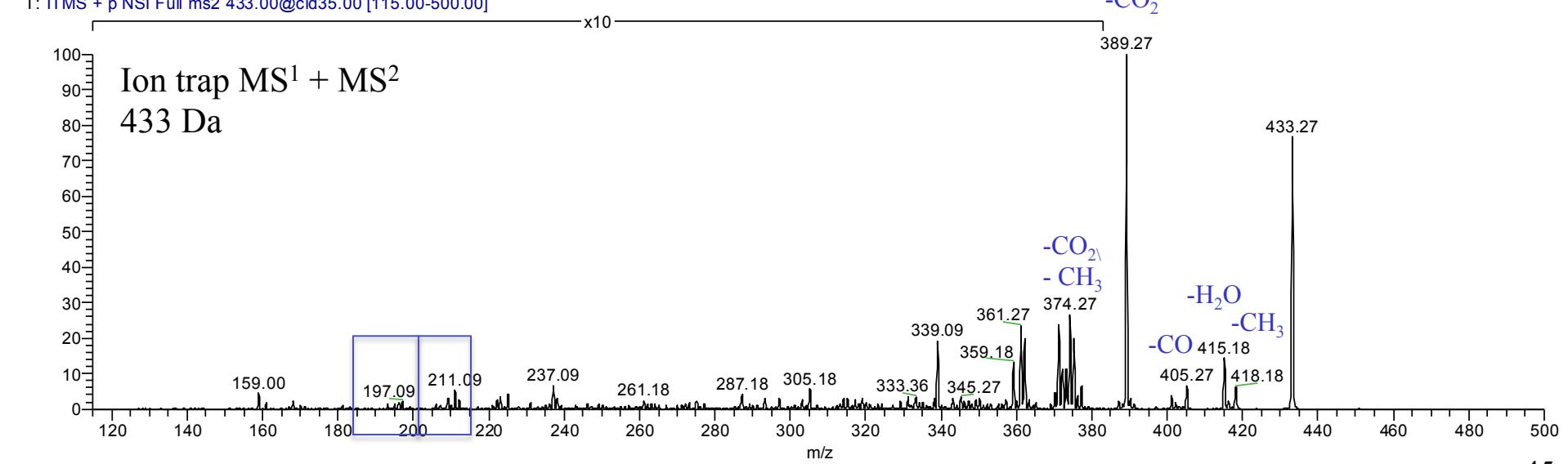


$$\text{Calc } [\text{M}+\text{H}]^+ = 433.1295$$

$$\text{Obs } [\text{M}+\text{H}]^+ = 433.1270$$

$$\Delta = -5.7 \text{ ppm}$$

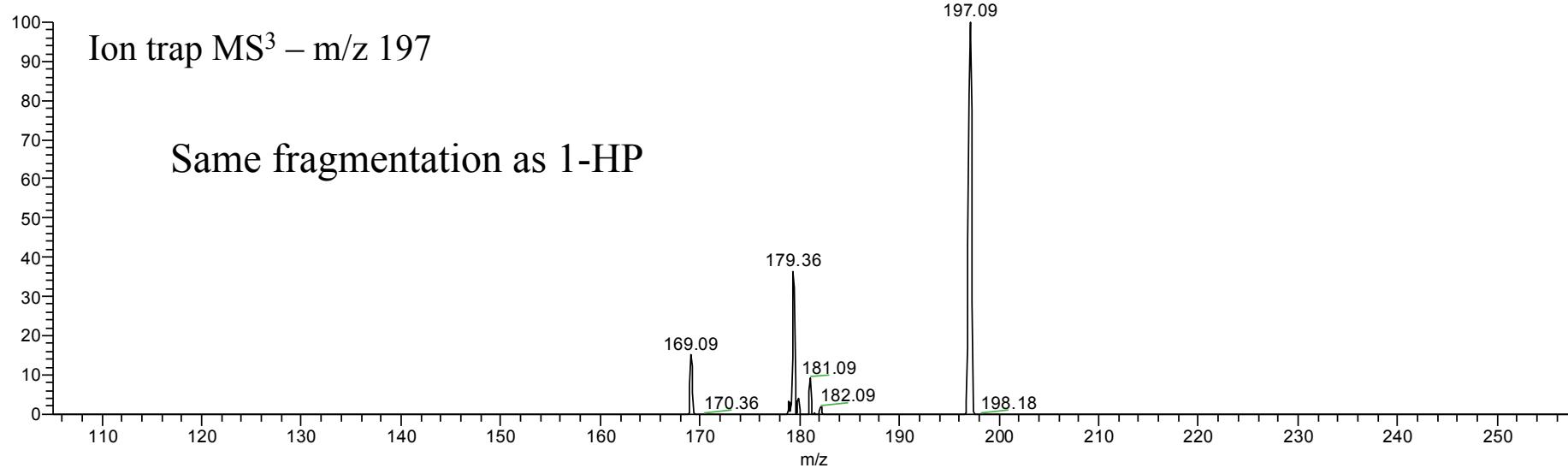
3-140Fr6_IT433 #1-109 RT: 0.00-0.62 NL: 2.70E2
T: ITMS + p NSI Full ms2 433.00@cid35.00 [115.00-500.00]



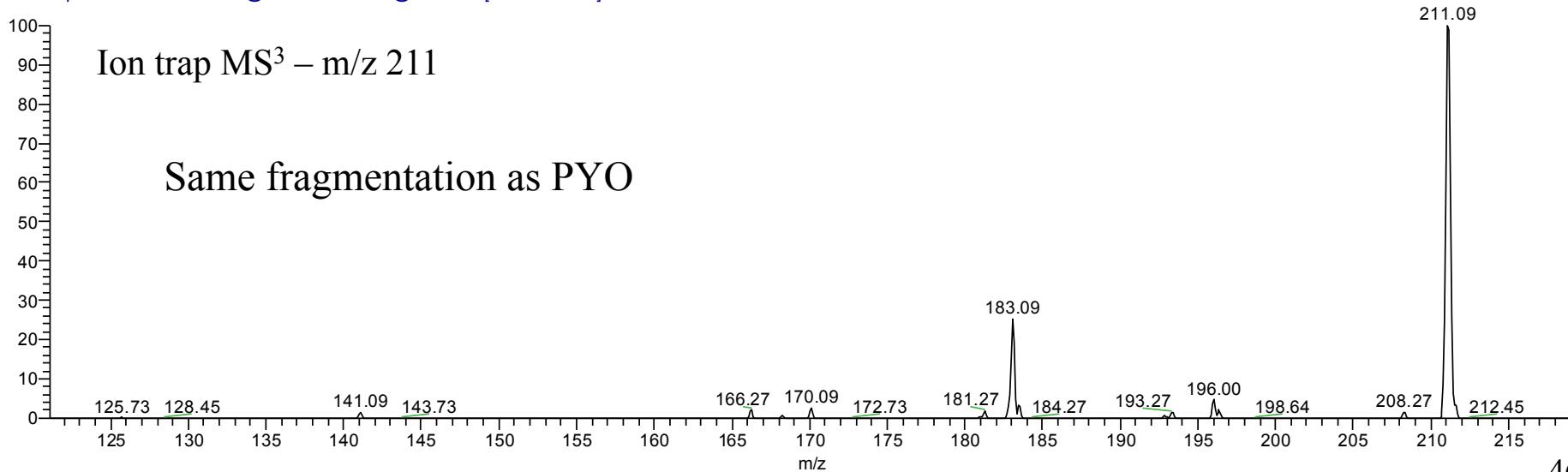
Phenazine-dimer (compound 10; m/z 433 Da)

EtOAc extract isolated from *A. fumigatus* - *P. aeruginosa* interaction

3-140Fr6_IT433_197 #1-132 RT: 0.00- V: 132 NL: 8.51E-2
T: ITMS + p NSI Full ms3 433.00@cid35.00 197.00@cid35.00 [50.00-500.00]



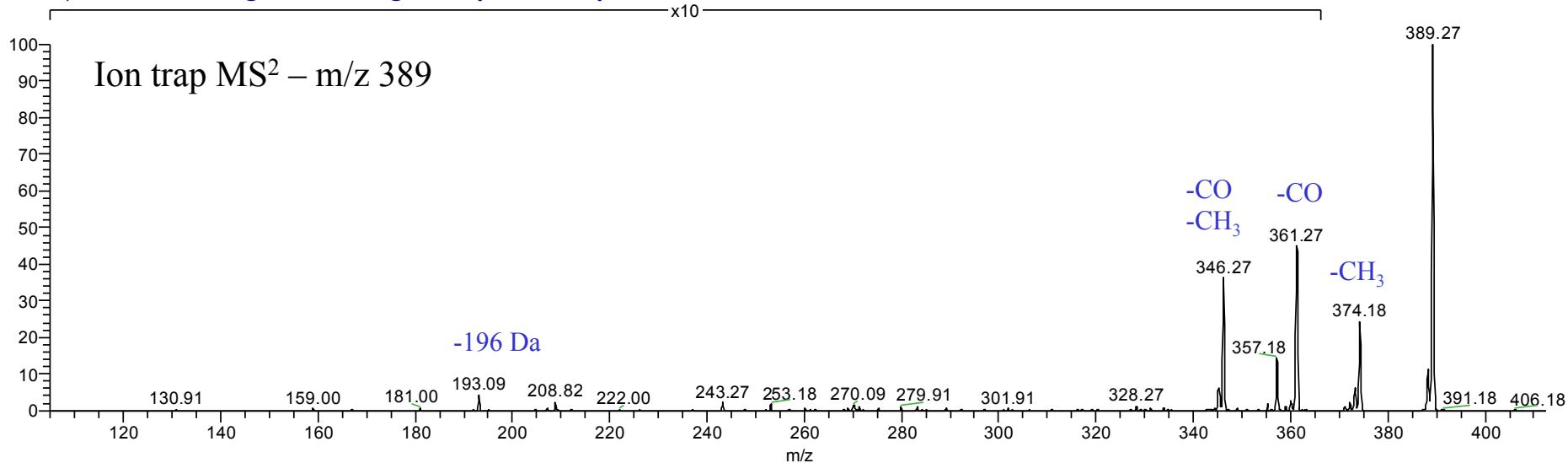
3-140Fr6_IT433_211 #1-172 RT: 0.00- V: 172 NL: 2.47E-1
T: ITMS + p NSI Full ms3 433.00@cid35.00 211.00@cid35.00 [55.00-500.00]



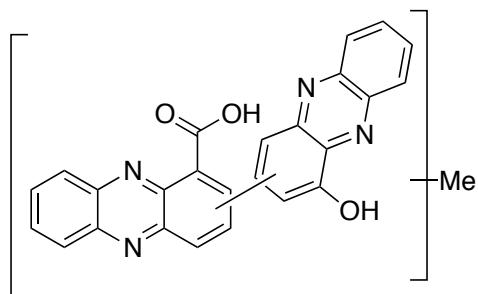
Phenazine-dimer (compound 10; m/z 433 Da)

EtOAc extract isolated from *A. fumigatus* - *P. aeruginosa* interaction

4-113Afu_outsideHPLCfr3_IT433_389 #5 T: 0.08-0.39 AV: 17 NL: 6.14E1
T: ITMS + p NSI Full ms3 433.20@cid35.00 389.00@cid35.00 [105.00-500.00]

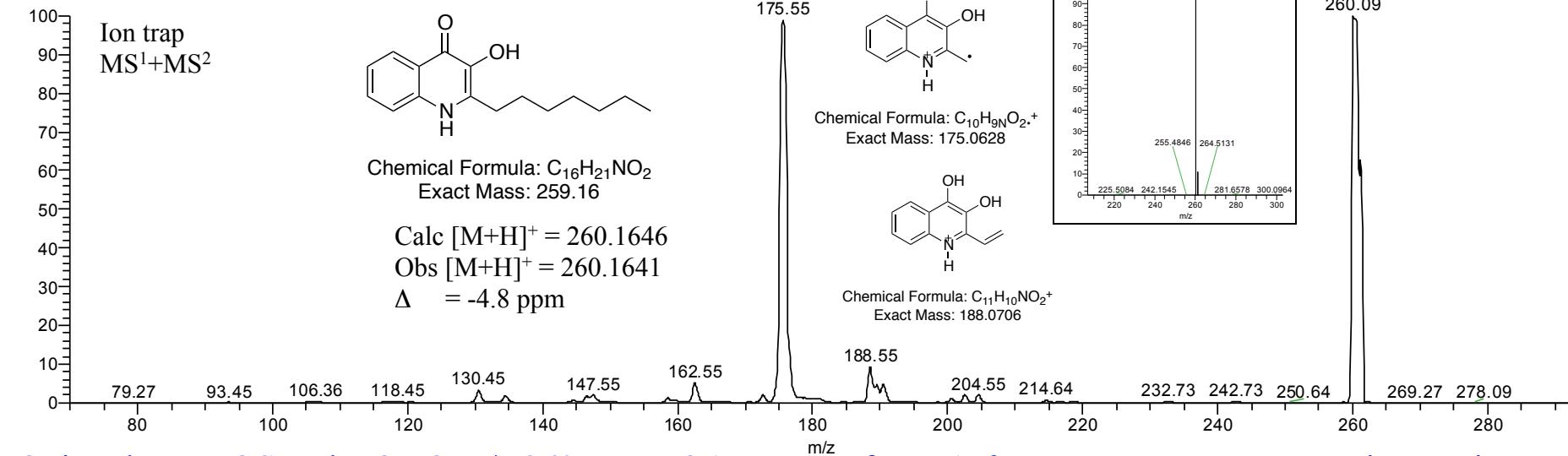


Putative structure



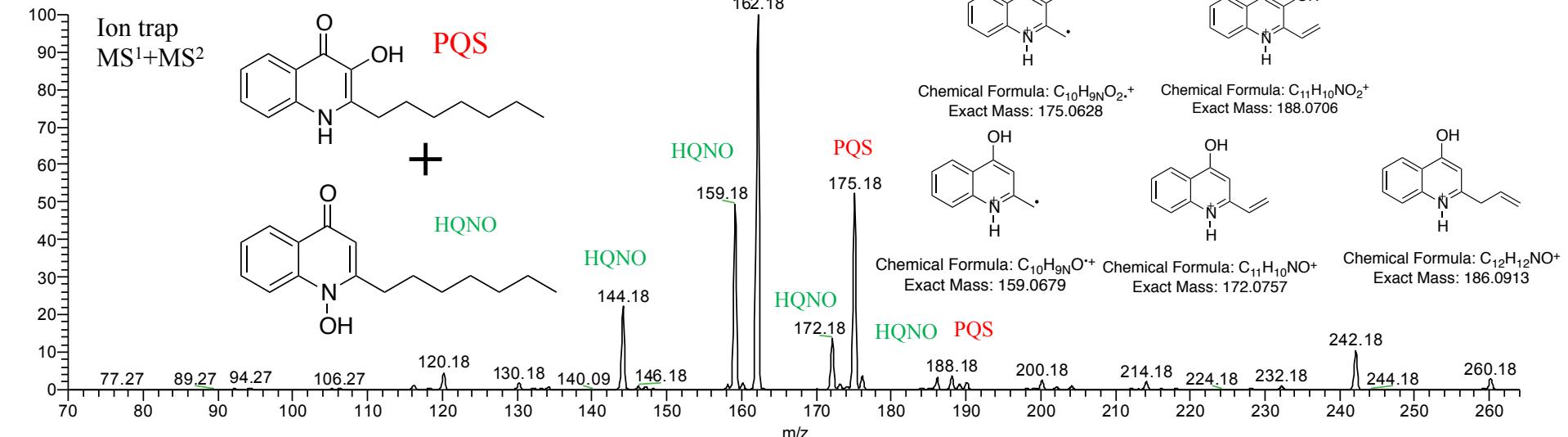
Quinolone PQS m/z 260 (Aldrich) (compound class 11)

quinolone PQS_IT_260 #45-100 RT: 0. AV: 56 NL: 3.46E5
T: ITMS + p NSI Full ms2 260.30@cid35.00 [70.00-1000.00]



Quinolones PQS and HQNO m/z 260 Da EtOAc extract from *A. fumigatus* – *P. aeruginosa* interaction

11825_PA14_afum_HPLC_3-140_12_IT_5 #1-337 RT: 0.00-1.01 AV: 337 NL: 4.26E1
T: ITMS + p NSI Full ms2 260.00@cid35.00 [70.00-600.00]

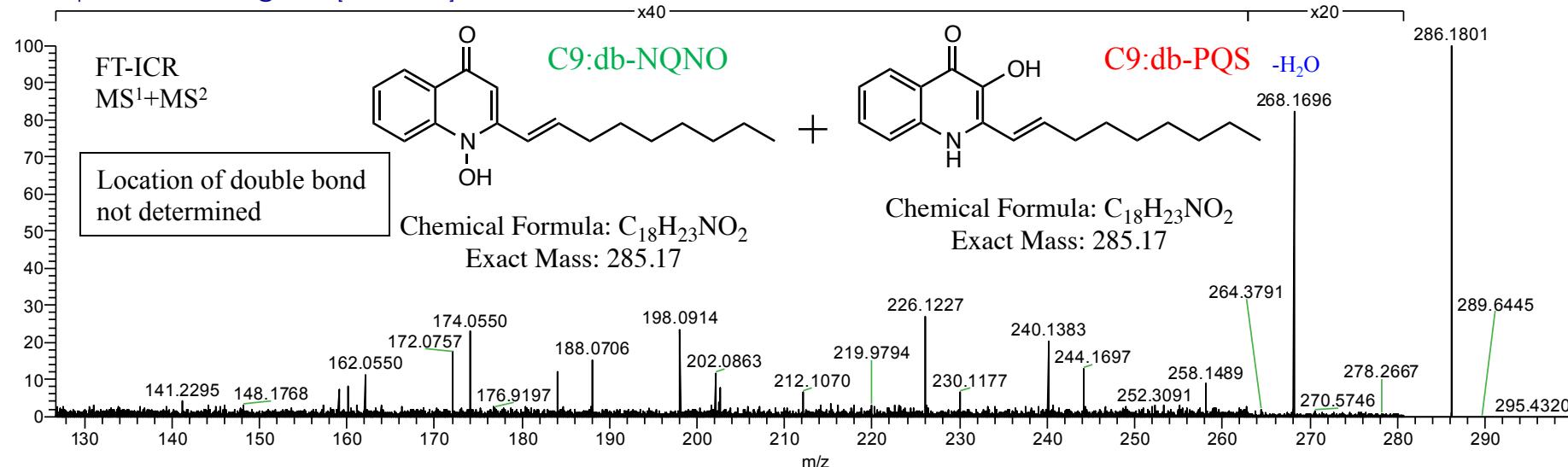


These spectra are consistent with published MS² data for both compounds.¹¹ For quinolone nomenclature see reference 5.

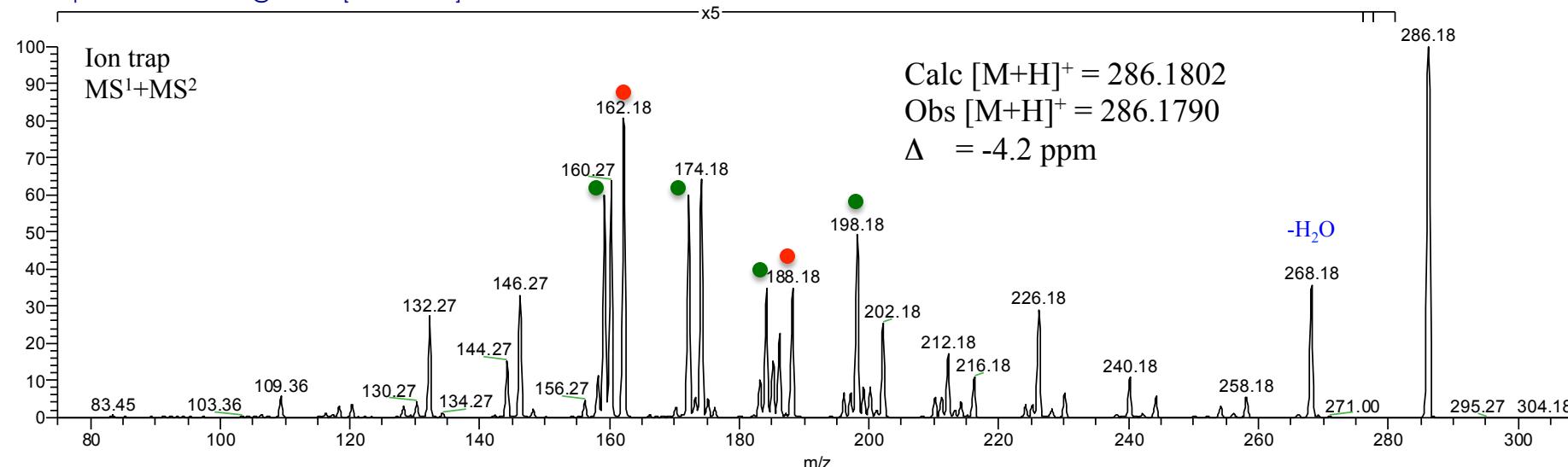
Quinolones m/z 286 Da (compound class 11)

EtOAc extract from *A. fumigatus* – *P. aeruginosa* interaction

090411AfumPA14_3_140_fr13_FT286 #1 T: 0.00-2.01 AV: 79 NL: 1.58E6
 T: FTMS + p NSI Full ms2 286.00@cid0.00 [75.00-500.00]



090411AfumPA14_3_140_fr13_IT286 #4- T: 0.01-1.57 AV: 541 NL: 2.98E5
 T: ITMS + p NSI Full ms2 286.00@cid35.00 [75.00-500.00]



For quinolone nomenclature see reference 5.

Rhamnolipid m/z 673 Da (Na^+ salt) (compound class 12) nBuOH extract from *A. fumigatus* – *P. aeruginosa* interaction

$[\text{M}+\text{Na}]^+$

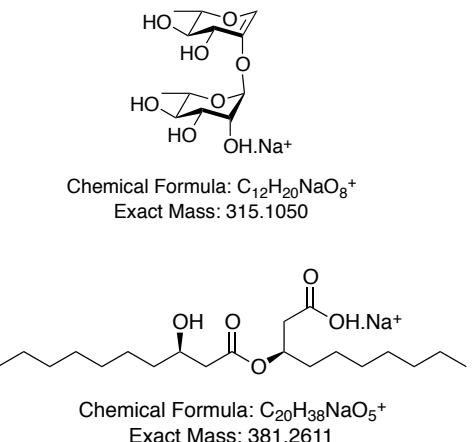
673.3742

$[\text{M}+\text{H}]^+$

383

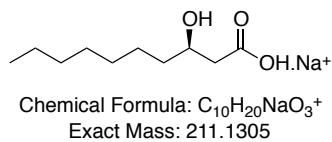
583.3251

721.3596



PA14control_Datadependents5 #18 RT: 175.00 V: 1 NL: 2.76E5
T: ITMS + c NS1d Full ms2 673.37@cid35.00 [175.00-1360.00]

Ion trap
MS² m/z 673



C10Na

211.23

C10-C10Na
- 292 Da

381.27

Rha-Rha-H₂O/Na
- 358 Da

315.12
Rha-C10Na
- 316 Da

333.16

357.19

414.33

Rha-C10-C10Na
- 146 Da

527.33

Rha-Rha-C10Na
- 170 Da

503.23

509.37

543.50

569.33

584.14

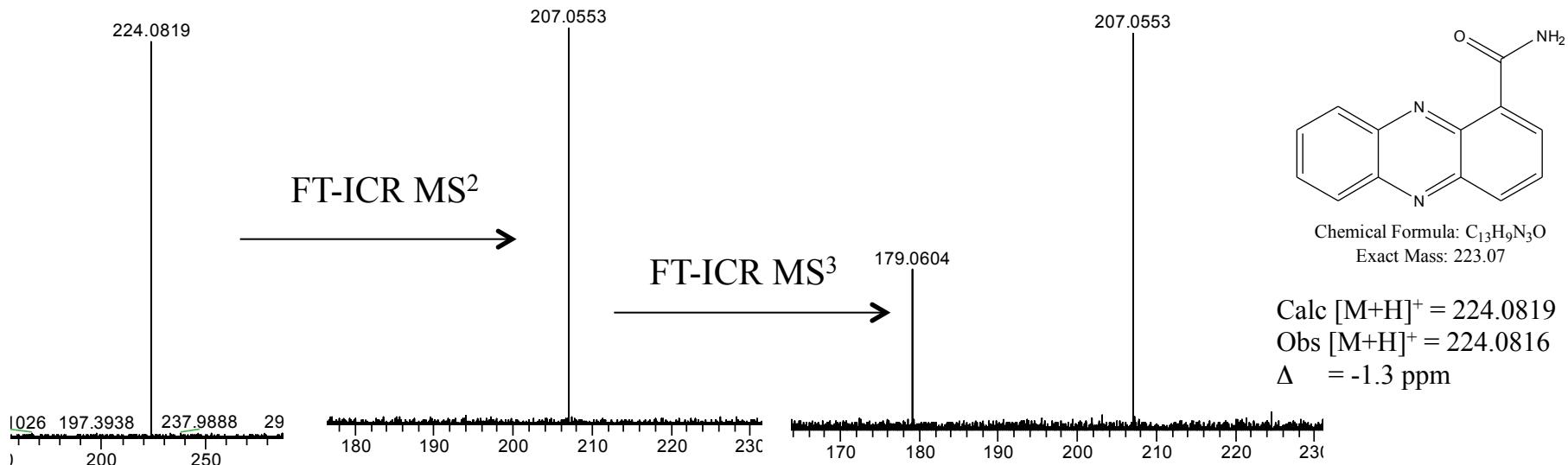
614.48

655.30

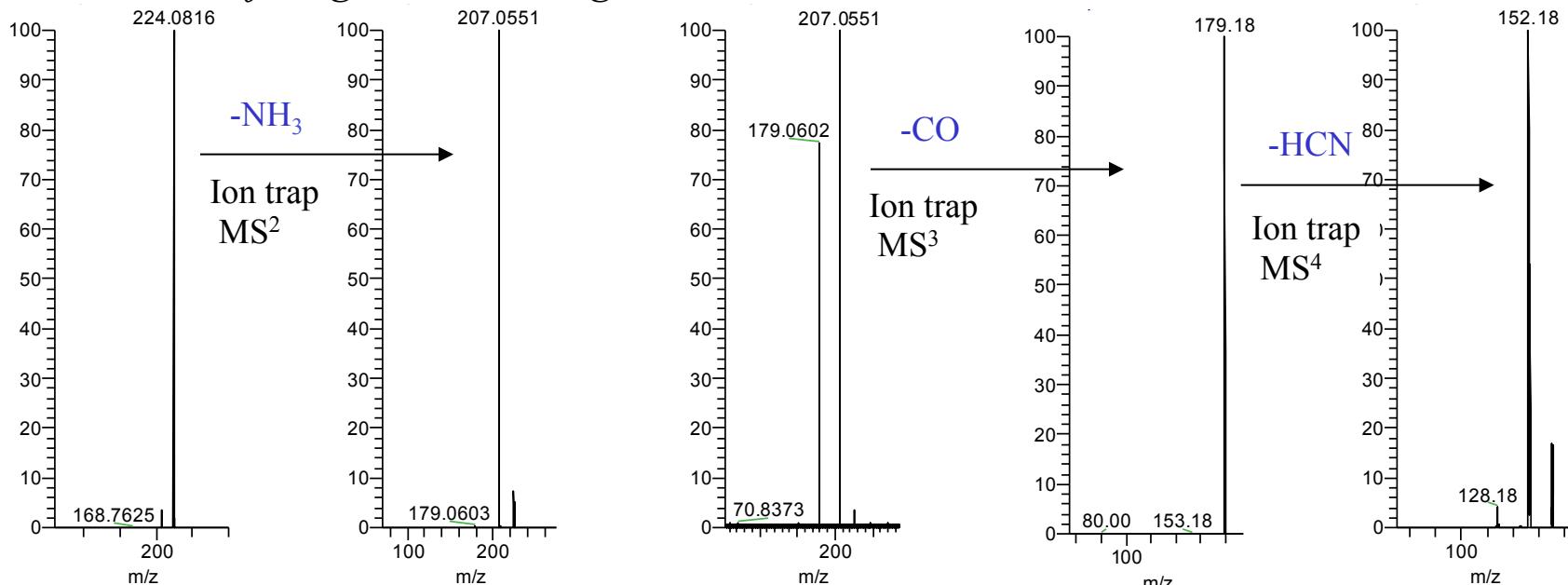
These spectra are consistent with published MS² data for rhamnolipids.¹² Stereochemistry shown is based on reported structural identification - for an overview of identified rhamnolipid congeners and nomenclature see ref 6.

Phenazine-1-carboxyamide (PCN; compound 13)

Commercial from Princeton Bioblocks



EtOAc extract *A. fumigatus* - *P. aeruginosa* interaction



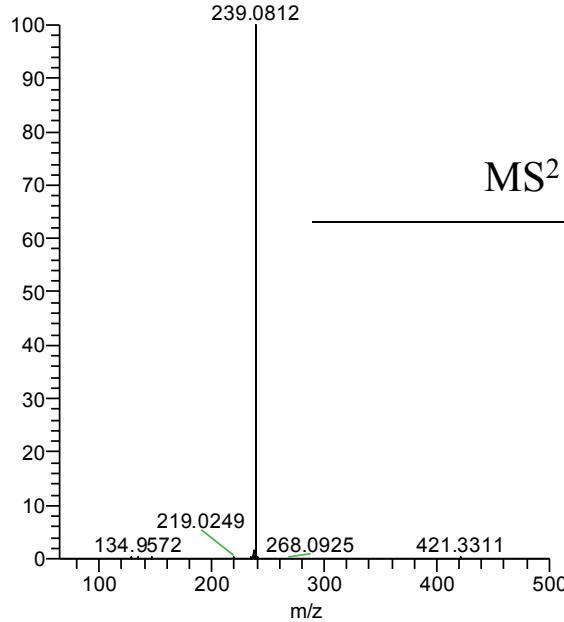
Fragmentation data in accordance with previous reports.⁷

5-N-methyl-phenazine-1-carboxylicacid (5-MPCA; compound 14)

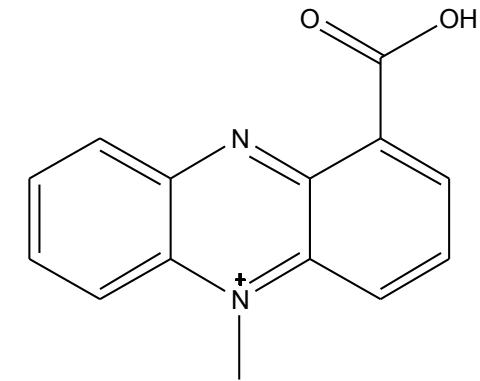
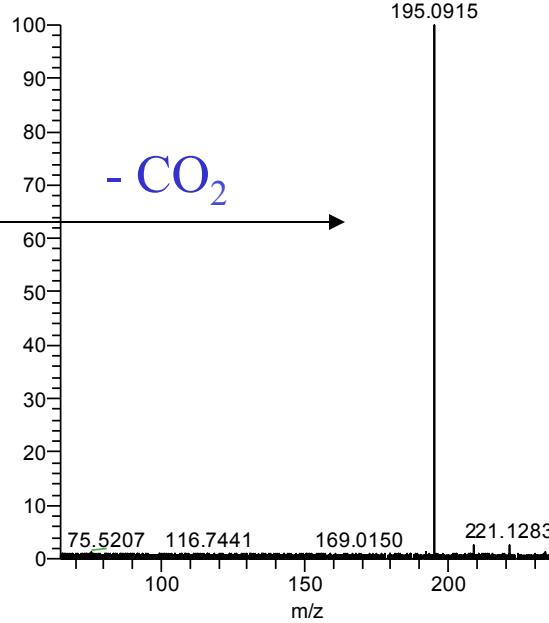
EtOAc extract from *A. fumigatus* - *P. aeruginosa* interaction

FT-ICR data

11825_PA14_afum_HPLC_3-140_4_Ftf_23^c T: 0.00 AV:
T: FTMS + p NSI Full ms2 239.00@cid0.00 [65.00-500.00]



11825_PA14_afum_HPLC_3-140_4_Ftf_23^c RT: 0.00
T: FTMS + p NSI Full ms2 239.00@cid35.00 [65.00-500.00]



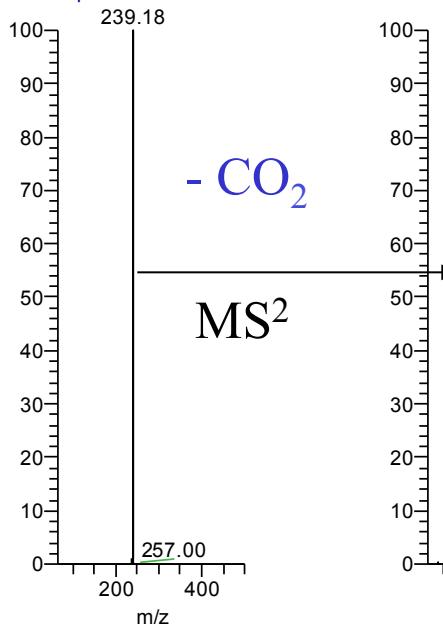
Chemical Formula: $C_{14}H_{11}N_2O_2^+$
Exact Mass: 239.08

Calc $[M]^+ = 239.0816$
Obs $[M]^+ = 239.0809$
 $\Delta = -2.9 \text{ ppm}$

5-MPCA (compound 14)

EtOAc extract from *A. fumigatus* - *P. aeruginosa* interaction

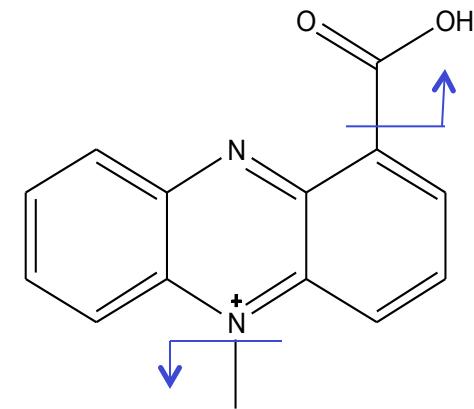
T: ITMS + p NSI Full ms2 23 ...



T: ITMS + p NSI Full ms2 23 ...



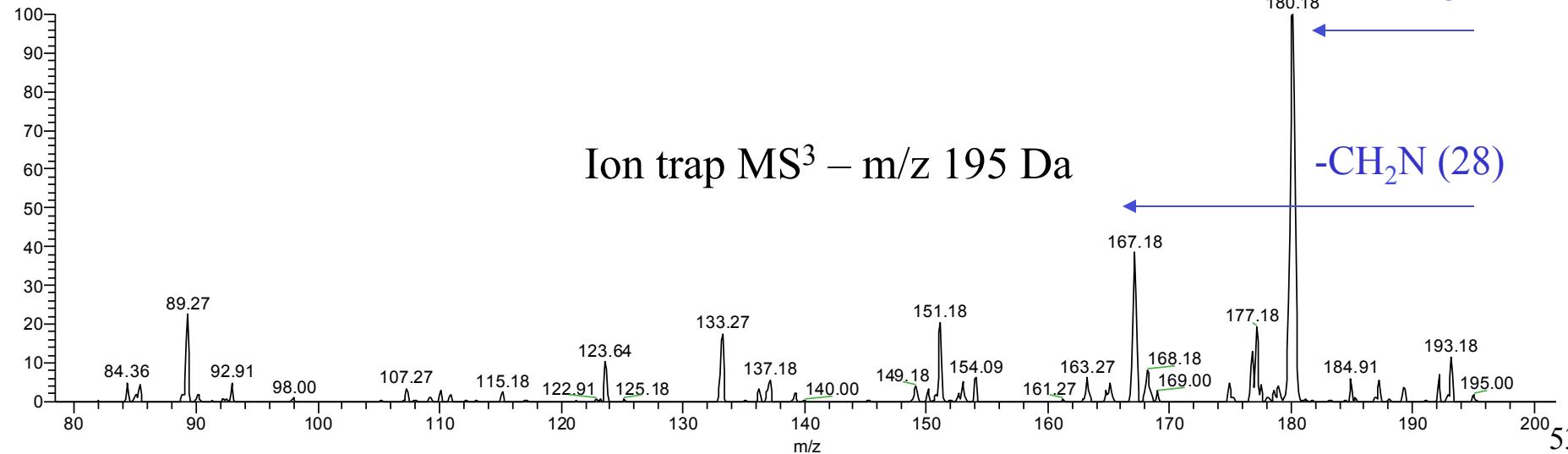
Ion trap MS²



Chemical Formula: C₁₄H₁₁N₂O₂⁺

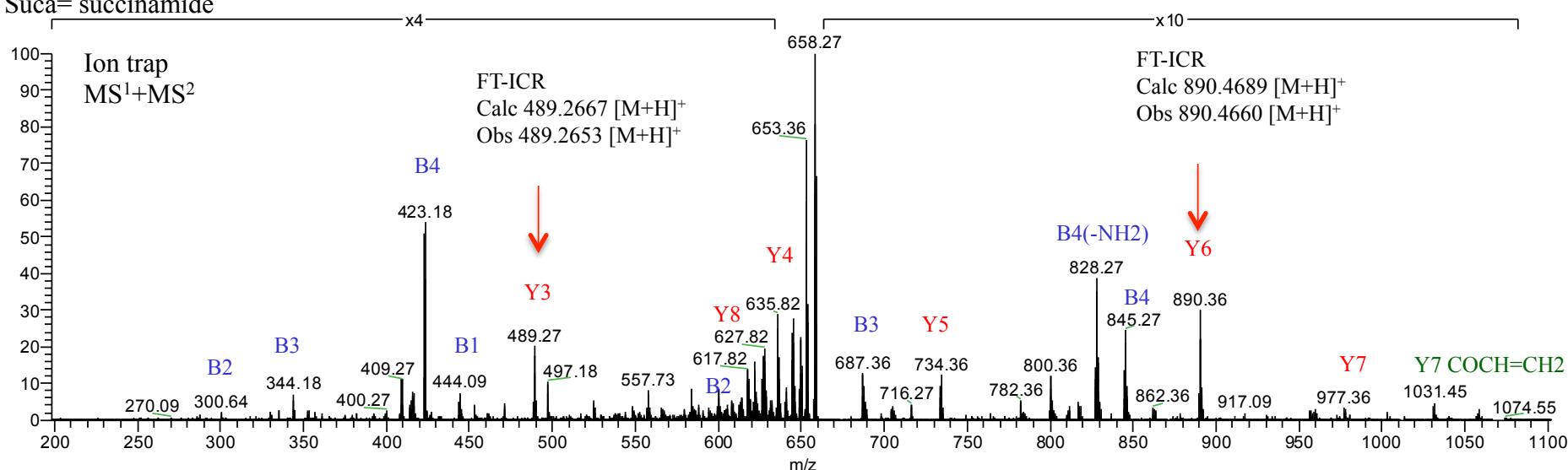
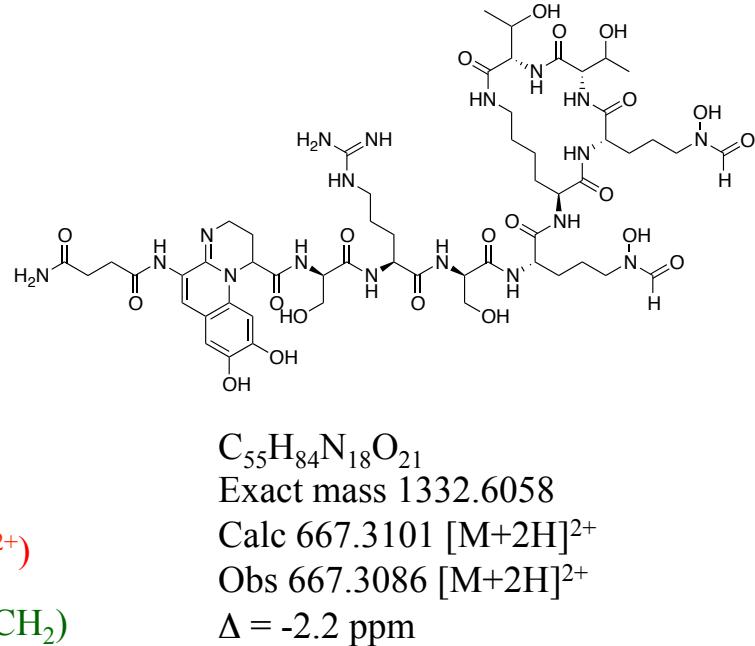
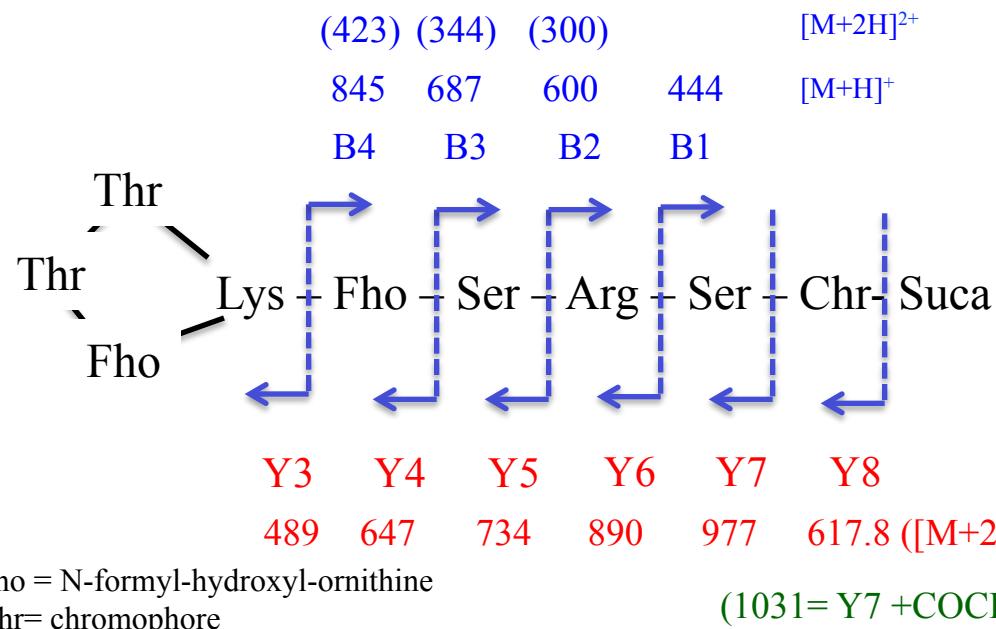
Exact Mass: 239.08

11825_PA14_afum_HPLC_3-140_4_IT_..._5_35 #1 RT: 0.00 AV: 1 NL: 6.09
T: ITMS + p NSI Full ms3 239.00@cid35.00 195.00@cid35.00 [50.00-500.00]



Pyoverdin E (compound 15)

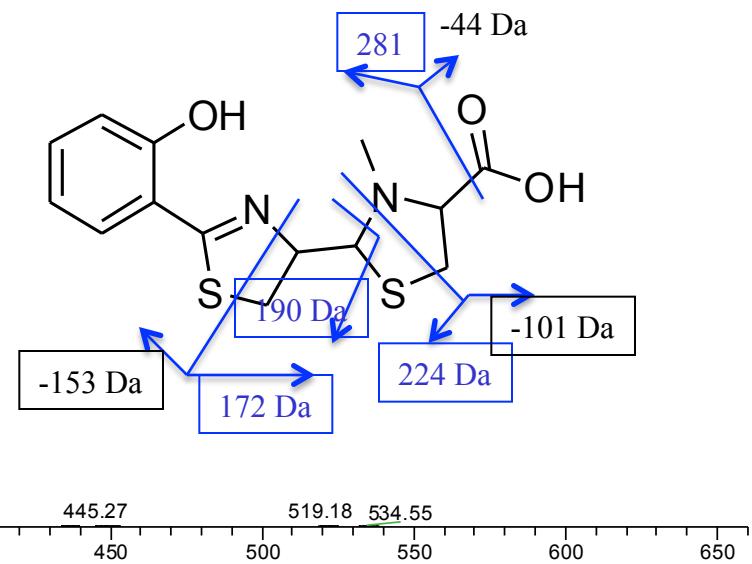
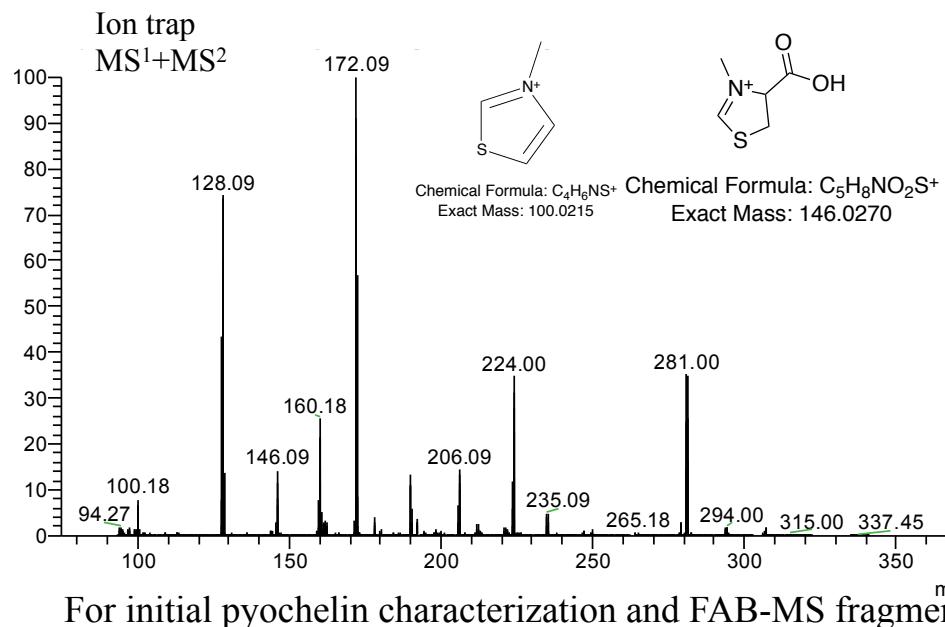
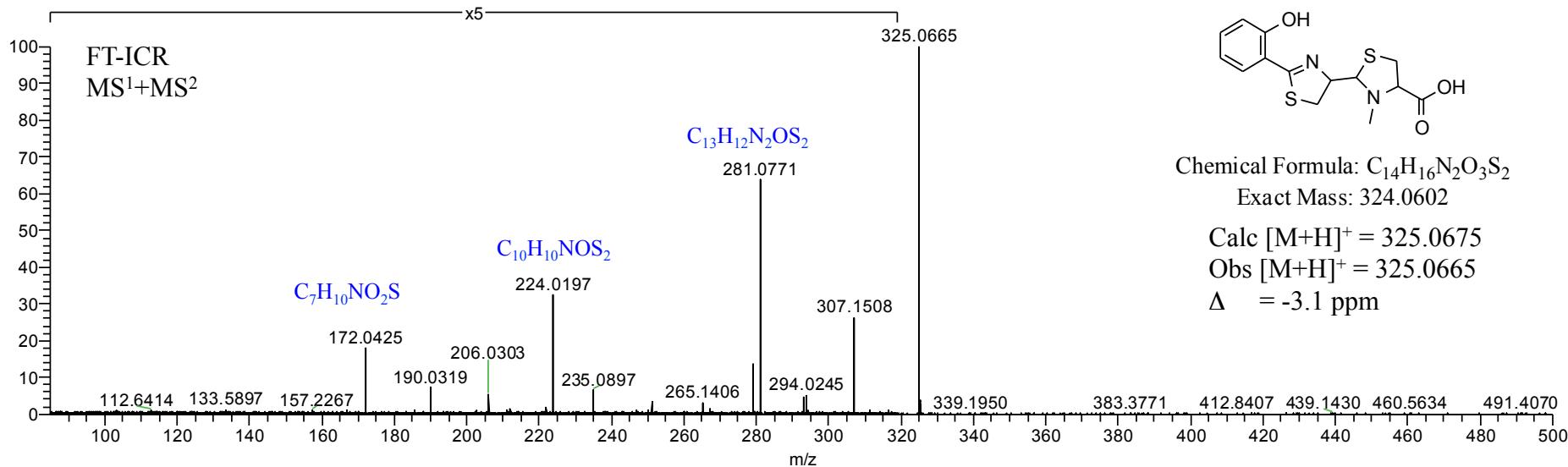
Water/FA extract from *A. fumigatus* – *P. aeruginosa* interaction



Fragmentation is in accordance with reported data and stereochemistry shown is based on reported structural data.^{13,14}

Pyochelin m/z 325 Da (compound 16)

$\text{H}_2\text{O}/\text{AcN}/\text{FA}$ extract from *A. fumigatus – P. aeruginosa* interaction

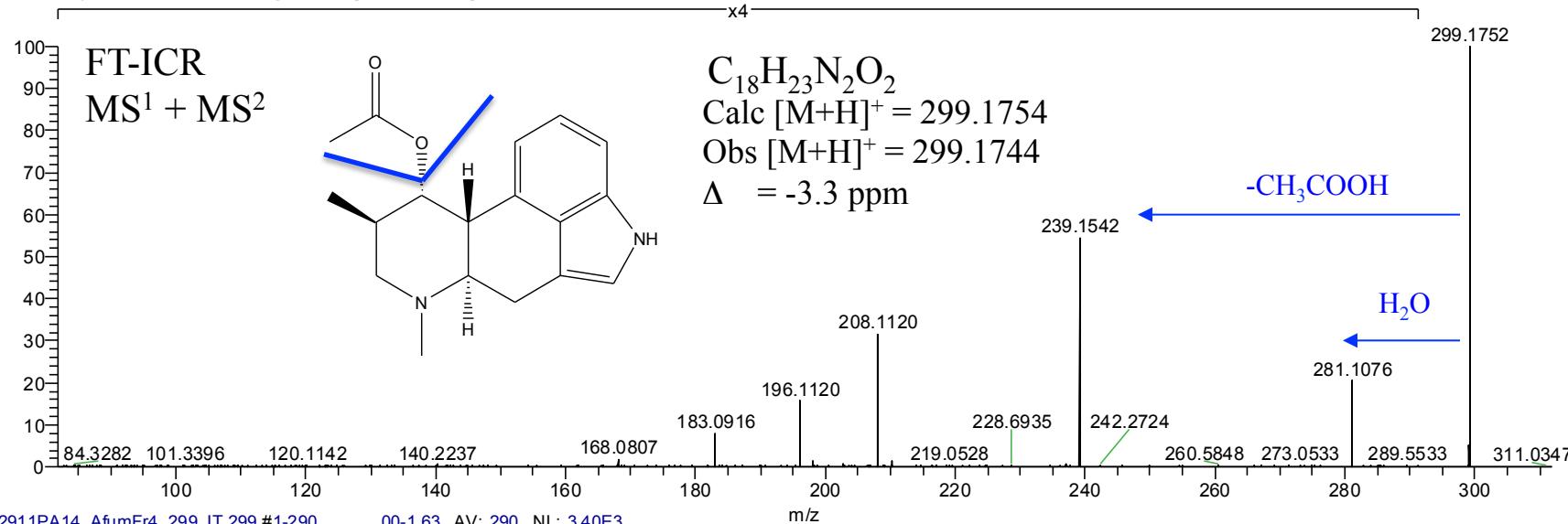


For initial pyochelin characterization and FAB-MS fragmentation see ref 15.

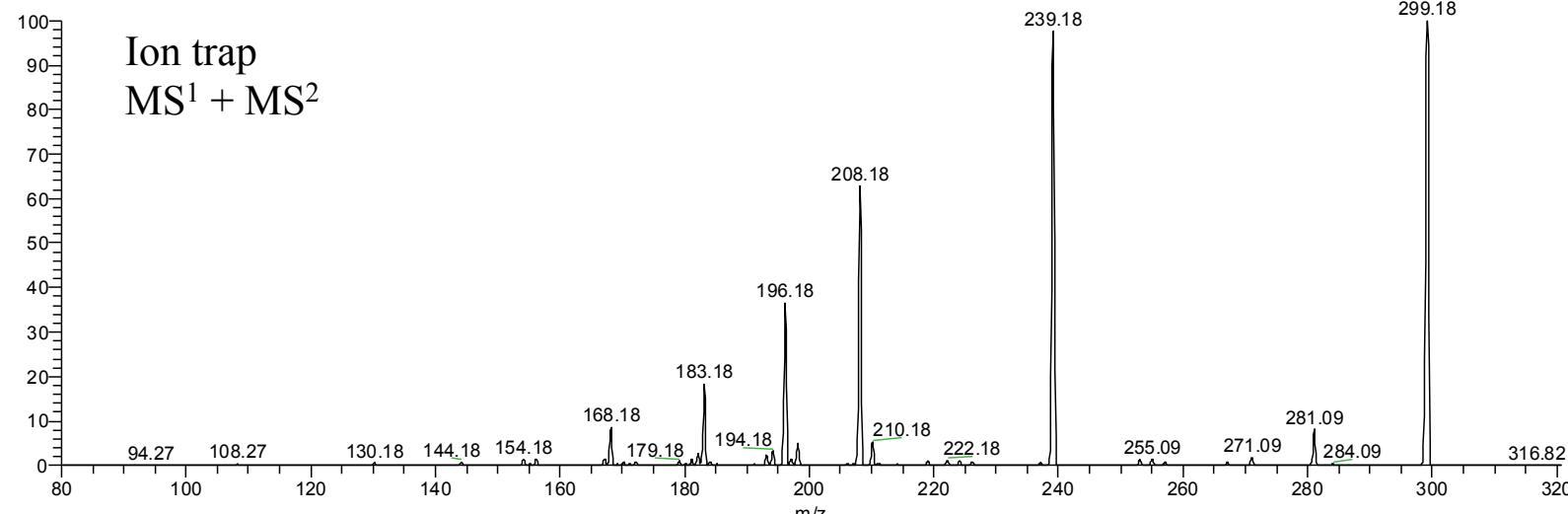
Fumigaclavine A

Detected in EtOAc extract of interaction between *A. fumigatus* and *P. aeruginosa*

82911PA14_AfumFr4_299_FT 299 #2-110 02-3.30 AV: 109 NL: 2.54E4
 T: ITMS + p NSI Full ms2 299.00@cid0.00 [80.00-600.00]



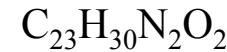
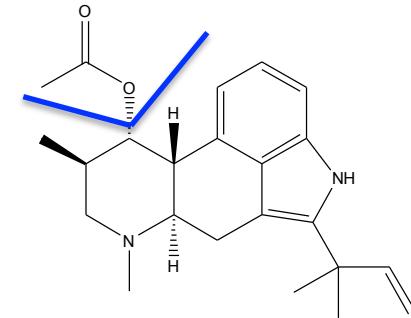
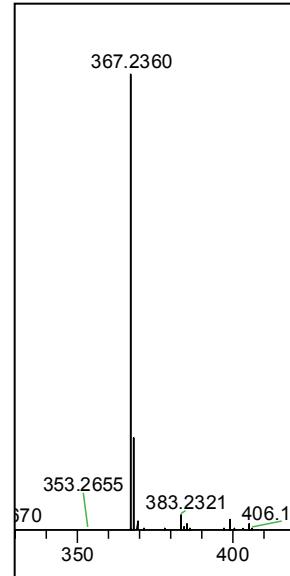
82911PA14_AfumFr4_299_IT 299 #1-290 00-1.63 AV: 290 NL: 3.40E3
 T: ITMS + p NSI Full ms2 299.00@cid35.00 [80.00-1400.00]



MS² fragmentation data is in accordance with published data.¹⁶

Fumigaclavine C

Detected in EtOAc extract of interaction between *A. fumigatus* and *P. aeruginosa*



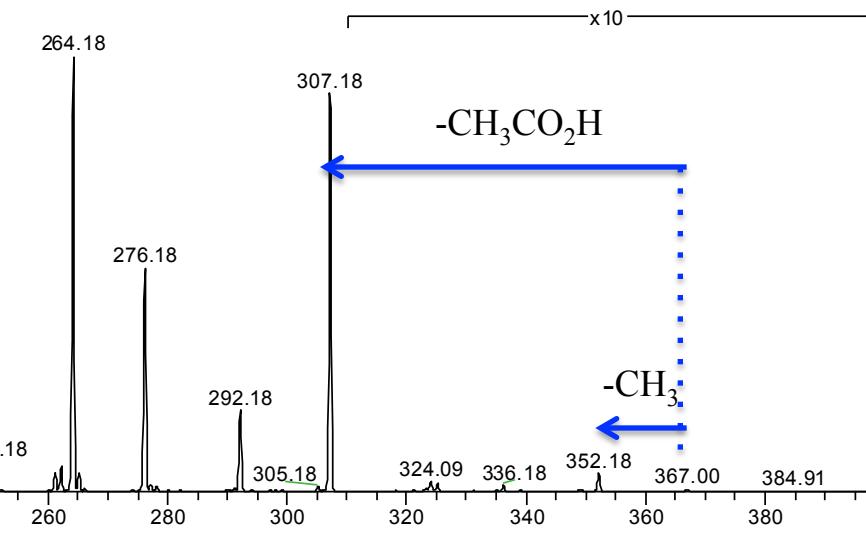
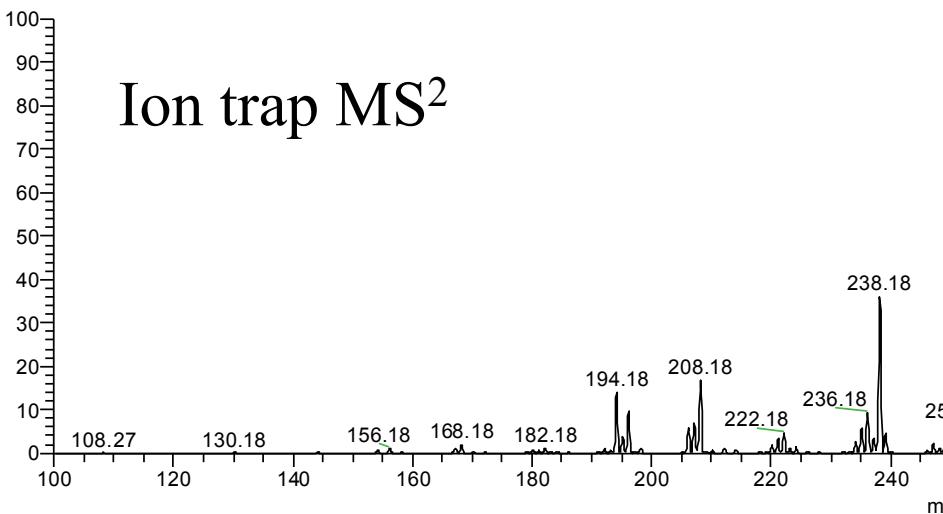
$$\text{Calc } [\text{M}+\text{H}]^+ = 367.2376$$

$$\text{Obs } [\text{M}+\text{H}]^+ = 367.2360$$

$$\Delta = -4.3 \text{ ppm}$$

11825_PA14_afum_HPLC_3-140_f9_IT_#1-313 RT: 0.00-0.93 AV: 313 NL: 4.21E4
T: ITMS + p NSI Full ms2 367.00@cid35.00 [100.00-600.00]

Ion trap MS²



MS² fragmentation data is in accordance with published data.¹⁶

d) Supplementary Information - References

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14. Demange P, *et al.* (1990) Bacterial siderophores: structure and NMR assignment of pyoverdins Pa, siderophores of *Pseudomonas aeruginosa* ATCC 15692 *Biol Metals* 3, 155-170.
15. Coxt CD, Rinehart KL, Moore ML, Cook JC. (1981) Pyochelin: Novel structure of an iron-chelating growth promoter for *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 78: 4256-4260.
16. Ostertag J. (2010) Nachweis und Vorkommen von *Aspergillus fumigatus*-Toxinen in Gras- und Maissilagen. Ph D dissertation, Munchen, Germany.